ViroSeq™ HIV-1 Genotyping System

Version 2

User's Manual

Applied Biosystems

For Research Use Only. Not for use in diagnostic procedures.

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1 Introduction

Chapter Overview

Introduction This chapter describes the conventions used in this manual, provides an overview of the ViroSeq™ HIV-1 Genotyping System, lists materials needed to use the system, and tells you how to get help from Applied Biosystems Technical Support.

In This Chapter The following topics are covered in this chapter:

Safety

Documentation User Attention Words

Five user attention words appear in the text of all Applied Biosystems user documentation. Each word implies a particular level of observation or action as described below.

Note Calls attention to useful information.

IMPORTANT Indicates information that is necessary for proper instrument operation.

CAUTION Cautions the user that a potentially hazardous situation could occur, causing injury to the user or damage to the instrument, if this information is ignored.

! WARNING ! Warns the user that serious physical injury or death to the user or other persons could result if these precautions are not taken.

! DANGER ! Indicates an imminently hazardous situation that, if not avoided, will result in death or serious injury.

Chemical Hazard Warning

! WARNING ! CHEMICAL HAZARD. Some of the chemicals used with Applied Biosystems instruments and protocols are potentially hazardous and can cause injury, illness or death.

- **Read and understand the material safety data sheets (MSDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.**
- **Minimize contact with and inhalation of chemicals. Wear appropriate personal protective equipment when handling chemicals (*e.g.*, safety glasses, gloves, or clothing). For additional safety guidelines consult the MSDS.**
- **Do not leave chemical containers open. Use only with adequate ventilation.**
- **Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.**
- **Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.**

Site Preparation and Safety Guide

A Site Preparation and Safety Guide is a separate document sent to all customers who have purchased a Applied Biosystems instrument. Refer to the Guide written for your instrument for information on site preparation, instrument safety, chemical safety and waste profiles.

About MSDSs

Some of the chemicals used with this instrument may be listed as hazardous by their manufacturer. When hazards exist warnings are prominently displayed on the labels of all chemicals.

Chemical manufacturers supply a current MSDS before or with shipments of hazardous chemicals to new customers and with the first shipment of a hazardous chemical after an MSDS update. MSDSs provide you with the safety information you need to store, handle, transport and dispose of the chemicals safely.

We strongly recommend that you replace the MSDSs in your files each time you receive one packaged with hazardous chemicals.

! WARNING ! CHEMICAL HAZARD. Be sure to familiarize yourself with the MSDSs before using reagents or solvents.

Ordering MSDSs

You can order free additional copies of MSDSs for chemicals manufactured or distributed by Applied Biosystems using the contact information below.

To order MSDSs...	Then...
Over the Internet	Go to our web site at: www.appliedbiosystems.com/techsupport a. Click on **MSDSs** b. Enter keywords (or partial words), or a part number, or the MSDSs Documents on Demand index number. c. Click on **Search** d. Click on the Adobe Acrobat symbol to view, print, or download the document, or check the box of the desired document and delivery method (fax or e-mail)
By automated telephone service from any country	Use “To Obtain Documents on Demand” on page 1-30.

<table>
<tr><th>To order MSDSs...</th><th>Then...</th></tr>
<tr><td>By telephone in the United States</td><td>Dial 1-800-327-3002, then press 1</td></tr>
<tr><td>By telephone from Canada</td><td><table><tr><th>To order in...</th><th>Then dial 1-800-668-6913 and...</th></tr><tr><td>English</td><td>Press 1, then 2, then 1 again</td></tr><tr><td>French</td><td>Press 2, then 2, then 1</td></tr></table></td></tr>
<tr><td>By telephone from any other country</td><td>See “To Contact Technical Support by Telephone or Fax” on page 1-26.</td></tr>
</table>

For chemicals not manufactured or distributed by Applied Biosystems, call the chemical manufacturer.

The ViroSeq HIV-1 Genotyping System

Intended Use

The ViroSeq HIV-1 Genotyping System is used for identifying mutations in the pol gene of the human immunodeficiency virus, type one (HIV-1). The entire Protease gene and approximately two thirds of the Reverse Transcriptase (RT) gene in the pol open reading frame are amplified (approximately 1.8 kilobases (kb)). This amplicon is subsequently used as a sequencing template to generate approximately 1.2 kb of sequence data.

Facilitates Study of Mutations and Viral Resistance

Genotypic analysis of this region of HIV-1 facilitates the study of the relationship between mutations and viral resistance to anti-retroviral drugs, specifically the protease and RT inhibitors.

The ViroSeq HIV-1 Genotyping System can be used to process samples with viral loads between 2000 and 750,000 copies per milliliter (cp/mL).

For Research Use Only

The ViroSeq HIV-1 Genotyping System is for research use only, not for use in diagnostic procedures.

Features of the ViroSeq HIV-1 Genotyping System

The ViroSeq HIV-1 Genotyping System:

- Includes the reagents needed to purify the viral RNA from human blood plasma.
- Uses a reverse transcription-polymerase chain reaction (RT-PCR) to generate amplified DNA from viral RNA.
- Incorporates the AmpErase® Uracil N-glycosylase (UNG) contamination control system to safeguard against false genotyping
- Uses BigDye™ terminator sequencing chemistry.
- Uses a single tube for the RT and PCR for speed and convenience.
- Sequences both strands of amplified DNA to increase accuracy.
- Contains a synthetic, noninfectious, positive RNA control including the entire protease gene and the 5´ 945 nucleotides (315 amino acids) of the RT gene.
- Provides the required MicroAmp® Reaction Tubes and Microcon® YM-100 microconcentrators.
- Includes ViroSeq HIV-1 Genotyping System Software.

- Uses either the ABI PRISM® 377 DNA Sequencer or the ABI PRISM® 310 Genetic Analyzer from Applied Biosystems.
- Is optimized for use on the GeneAmp® PCR System 9600 and 9700 thermal cyclers from Applied Biosystems.

The Genotyping Process

The process of HIV-1 genotyping with the HIV-1 Genotyping System has five main stages:

Stage	Process
1	Isolating the HIV particles from plasma samples followed by the purification of viral RNA
2	Performing a reverse transcription of the HIV genome using a single primer
3	Performing PCR amplification of the protease and RT genes from the cDNA made in the reverse transcription reaction
4	Direct sequencing of the PCR amplification product using six or seven custom sequencing mixes
5	Identifying mutations in the protease or RT genes using the ViroSeq™ HIV-1 Genotyping System Software This process includes: ♦ Assembling the multiple sequences into a single contiguous sequence ♦ Manual reviewing and editing of the assembled sequence in comparison to a reference sequence, pNL4-3 strain of HIV-1, recommended by the AIDS Clinical Trials Group (ACTG) ♦ Identifying mutations known to be related to drug resistance mutation and novel mutations with no previously identified effects

RNA Purification

Viral RNA is purified from HIV particles that are isolated from plasma by centrifugation.

Reverse Transcription

The target for reverse transcription is the HIV-1 RNA prepared from plasma. The procedure is carried out using a single primer and the enzyme murine leukemia virus (MuLV) reverse transcriptase. This reaction produces a mixture of single-stranded cDNA fragments of varying length which represent the entire HIV-1 pol gene.

PCR

The cDNA products from the RT reaction are amplified. The resulting amplicon encompasses the entire HIV protease gene, and the 5´ end of the RT gene. The PCR procedure employs AmpliTaq Gold® for high specificity and efficiency and AmpErase UNG anticontamination chemistry to eliminate false genotyping.

For more information about the PCR procedure, see “ViroSeq HIV-1 Genotyping Process Summary Diagram” on page 1-8.

Sequencing

The PCR products are sequenced using custom primers formulated with the BigDye Terminator sequencing chemistry. The sequencing products are analyzed on an ABI PRISM 310 Genetic Analyzer or 377 DNA Sequencer. DNA basecalling is performed by the DNA Sequencing Analysis software.

For more information about sequencing chemistry, see the Automated DNA Sequencing Chemistry Guide (P/N 4305080).

Data Analysis

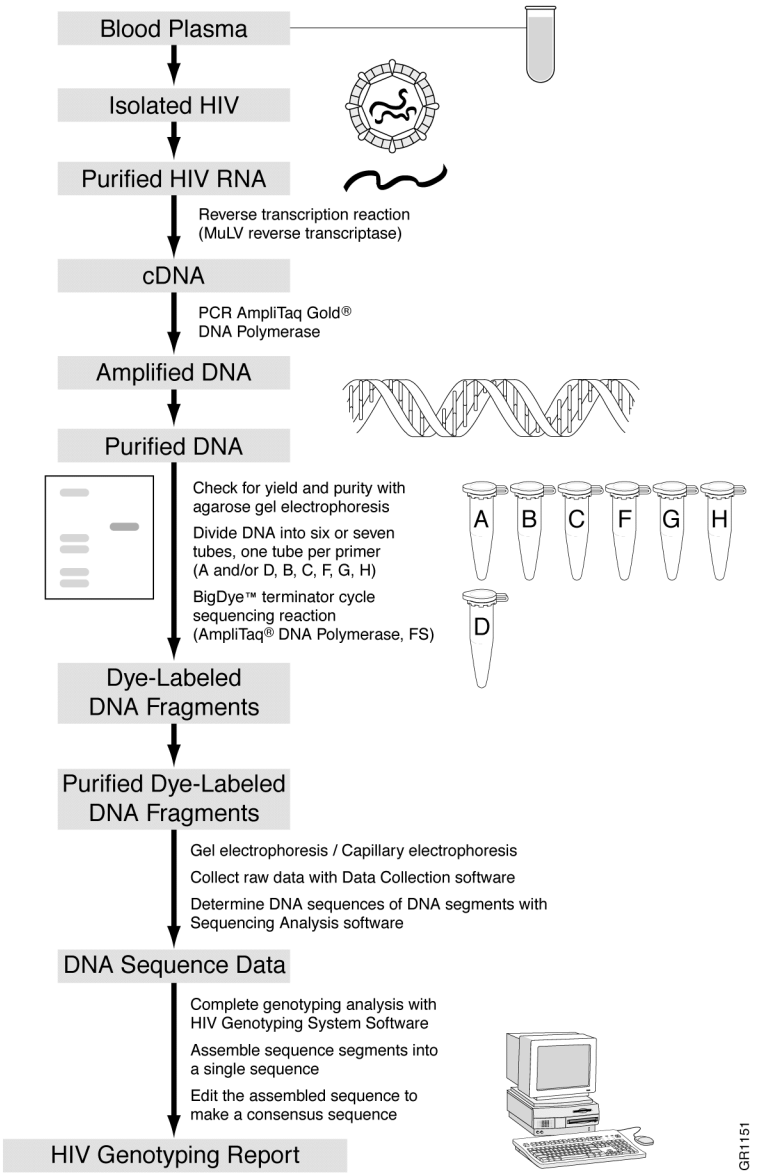
The ViroSeq HIV-1 Genotyping System Software automatically imports the sequence data from the DNA Sequencing Analysis software, and assembles the six or seven sequence segments into a single sequence, which is then compared to the reference strain.

After editing, a ViroSeq HIV-1 Genotyping System Report of the data is generated.

For more information about the ViroSeq HIV-1 Genotyping System Software, see page 1-12.

ViroSeq HIV-1 Genotyping Process Summary Diagram

The following diagram summarizes the HIV genotyping process:

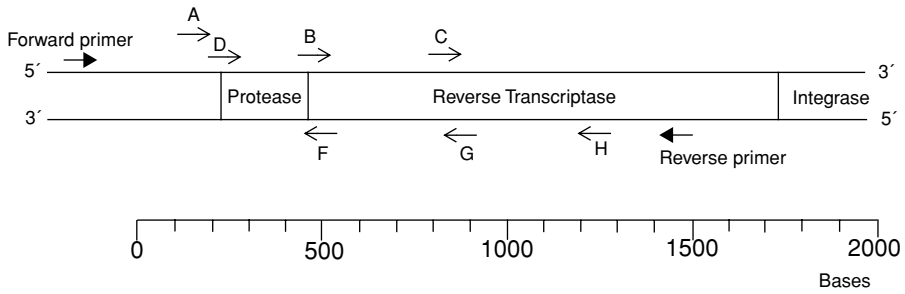


ViroSeq HIV-1 Genotyping System Components

Three Types of Primers are Provided

Three types of oligonucleotide primers are provided, preformulated, with the HIV-1 Genotyping System:

- Single primer for reverse transcription
- Sequence-specific primers for PCR (“forward” and “reverse” in the figure below)
- Sequence-specific primers for cycle sequencing (A, B, C, D, F, G, and H in the figure below)



Sequence Generated

The seven HIV-1 sequence-specific primers hybridize to the PCR product, as shown in the figure above. A sequence in both directions for all regions improves the accuracy of the data analysis.

Primers A and D

Why Primers A and D Were Created

Two primers (A and D) have been provided for sequencing the 5´ end of the PCR product. Due to known sequence polymorphism upstream of the protease gene, it has been necessary to design these two different sequencing primers.

Note If throughput is not an issue for your laboratory, you may choose to use both primers for every sample.

About Primers A and D

The following table describes the two primers:

Primer	Description
A	This is the preferred primer because it will provide the forward sequence of the entire protease gene. Use primer A first, because it works well with the majority of sequences. **If...** / **Then...** primer A does not give good results / repeat the procedure using primer D.
D	This primer extends more efficiently than primer A for some sequences. The disadvantage of primer D is that the first 4 to 8 codons of the protease gene are not sequenced in the forward direction.

Enzymes

The following table describes the four different enzymes provided with the HIV-1 Genotyping System:

Enzyme	Description
MuLV reverse transcriptase	Produces single-stranded cDNA from viral RNA
AmpliTaq Gold DNA Polymerase	♦ Generates double-stranded DNA fragments from the cDNA ♦ Amplifies the double-stranded DNA ♦ Provides hot-start for improved PCR performance
AmpliTaq® DNA Polymerase, FS	Creates extension products during the cycle sequencing reaction
AmpErase Uracil N-glycosylase (UNG)	Cleaves uracil-containing PCR products, reducing contamination from previous PCR reactions

RNase Inhibitor

Included with the HIV-1 Genotyping System is an RNase inhibitor that protects RNA against degradation by RNases. For more information about how to prevent RNA degradation, see page 2-2 and Appendix B, “Preventing RNA Degradation.”

Controls

About the Controls

Positive and negative controls are provided with the HIV-1 Genotyping System.The controls are introduced into the genotyping procedure in place of viral RNA at the RT step and are processed the same way as the HIV-1 RNA samples from blood plasma.

Positive and Negative Controls Described

Control	Description
Positive RNA Control	The positive control (RNA Control, PRT/5´RT) allows the effectiveness of the procedure to be monitored. A 2.8-kb fragment of RNA contains the entire protease gene and the 5´ end of the reverse transcriptase gene. In the RT-PCR reactions, a 1.8-kb product is generated. To prevent contamination of reagents, do not store the positive RNA control in the RT-PCR module. **Note** The positive RNA control is not infectious. **IMPORTANT** Keep the positive RNA control on ice during use.
Negative Control	The RNA Diluent, supplied with the HIV-1 Genotyping System, is used as a negative control as well as being used to dilute RNA samples. The negative control provides a check for the contamination of any reagents used in the procedure.

ViroSeq HIV-1 Genotyping System Software

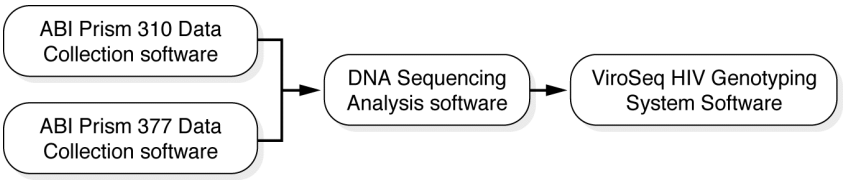
Overview

Three different types of software are used with the HIV-1 Genotyping System:

- Data Collection software for an ABI PRISM® instrument
- ABI PRISM® DNA Sequencing Analysis software
- ViroSeq HIV-1 Genotyping System Software

Data Flow Diagram

The flow of data between the different software applications is shown below.



The data from the Data Collection software are analyzed by the DNA Sequencing Analysis software and automatically imported into the HIV-1 Genotyping System Software.

You can run the HIV-1 Genotyping System Software on the collection computer or export the data to a separate computer.

Data Collection Software

The Data Collection software should already be installed on your computer, but you will need to configure it before running your samples (see Chapter 5, “Using the 310 Genetic Analyzer,” and Chapter 6, “Using the 377 DNA Sequencer.”).

Note The Data Collection software is different for the ABI PRISM 310 and ABI PRISM 377 instruments.

DNA Sequencing Analysis Software

The DNA Sequencing Analysis software is the same for the ABI PRISM 310 Genetic Analyzer and the ABI PRISM 377 DNA Sequencer. It was supplied with your instrument and should already be installed on your computer.

For more information about this software, see the *ABI PRISM DNA Sequencing Analysis Software User's Manual* (P/N 903564).

ViroSeq HIV-1 Genotyping System Software

About the HIV-1 Genotyping System Software

The ViroSeq HIV-1 Genotyping System Software is provided on a CD. It is used with the DNA Sequencing Analysis software and will analyze data from either instrument.

What the HIV-1 Genotyping System Software Does

The HIV-1 Genotyping System Software:

- Assembles the six or seven sequences obtained from a single plasma sample into a "project."
- Compares the overlapping segments to each other and to the sequence of the HIV-1 pNL4-3 reference strain.
- Allows you to examine the electropherograms of the sequence segments and assembled sequence, and then edit the assembled sequence to eliminate regions of poor quality data and correct for basecalling errors. This produces a consensus sequence.
- Automatically analyses the consensus sequence to show the "position of interest," defined as the following options:
  - Base mismatches between sequence segments
  - Multibase positions (those that indicate detectable levels of more than one genotype in the HIV population of the plasma sample)
  - Discrepancies between the consensus sequence and reference sequence
  - Base mutations that result in amino acid changes
  - Base mutations that correlate with base mutations reported to confer resistance to specific drugs
  - Bases that you have edited
- Generates a genotype report.

Saving Files with a FASTA File Format

If you want to compare two or more assembled or consensus sequences, the HIV-1 Genotyping System Software will allow you to save your files with a FASTA file format, which you can export to other sequence comparison programs.

Sample Naming

Sample Naming Requirement

IMPORTANT The ViroSeq HIV-1 Genotyping System Software uses the sample name to group sequencing data for a given plasma sample. All information to the left of the open, square bracket ([) must be identical for all sequencing files from the plasma sample. The bracket, and any information to the right of the bracket, is ignored by the software with regard to grouping, and may be used for comments, such as the date and the primer name.

Sample Naming Example

For the ViroSeq HIV-1 Genotyping System Software to recognize the HIV data files created by the Data Collection software, the samples loaded onto the instrument may be named using the ACTG 320 file-naming conventions.

The conventions specify that the following information is provided in the sample name, in the order given:

Name Part	Example
Sample identification number	630417L
Date of visit to the lab	021599
Lab code number	Not included in this example
Open square bracket	[
Primer letter	A
Date of sample analysis	021699

For the example given in the table, the full sample name would be: 630417L021599[A021699

Projects

Samples with the same name to the left of the square bracket are automatically grouped together by the HIV-1 Genotyping System Software into a single project. Samples in a project are assembled by the software into a single sequence.

A prerequisite for grouping particular samples into a project is that they are all saved in the same run folder by the Data Collection software. One run folder is created for each sample sheet filled out in the Data Collection software. Therefore, when you fill out the sample sheet, take into consideration the sample data that you want to include in a given project.

Multibase Analysis

Plasma samples may contain multiple strains of HIV, so it is possible that more than one genotype may be present in a single sample. In such cases, some base positions will have more than one nucleotide present (these positions are referred to as multibase positions).

During analysis, the HIV-1 Genotyping System Software identifies multibase positions based on a 30% threshold level. This means that if the peak height of a second nucleotide at any position is present at 30% or greater of the peak height of the main nucleotide, that position will be labeled as a multibase.

Conventional multibase nomenclature based on IUB codes are used to label the multibases. For more information about IUB Codes, see Appendix E, “IUB Codes.”

Computer Requirements

To run the HIV-1 Genotyping System Software, you will need:

- A Power Macintosh® computer, or later, using Macintosh® system software (Mac OS) 8.0 through 9.0 (or equivalent), and running at 150 mHz or higher.
- At least 64 MB of RAM, with 32 MB available
- At least 500 MB of hard disk memory available
- A CD-ROM drive
- A color printer

Materials and Equipment

Contents of the HIV-1 Genotyping System

The HIV-1 Genotyping System includes four modules, which contain the main consumable materials needed to perform the sample preparation, RT-PCR, sequencing, and quality control for 48 reactions.

For more information about these modules, see “Modules and Storage Conditions” on page 1-17.

The contents of this HIV-1 Genotyping System (P/N 4315425) are listed below.

Materials	Part Number
HIV Sample Prep Module RUO	4306027
ViroSeq™ HIV-1 RT-PCR Module RUO, version 2	4314888
HIV Sequencing Module RUO	4305611
HIV RNA Control Module RUO, version 2	4315236
MSDS, Formamide Recrystallized	L0044
MSDS, HIV Sample Prep	4306194
MSDS, DTT 100mM	4315270
Microcon YM-100 microconcentrators	4312669
MicroAmp® Tubes	M0035

HIV-1 Genotyping System Software

The HIV-1 Genotyping System Software is sold separately.

Item	Part Number
ViroSeq HIV-1 Genotyping System Software v2.x	4307816

Parts Sold Separately

The following components are sold as part of the complete kit but can also be purchased individually.

Note The ViroSeq RT-PCR module is not sold separately.

Parts Sold Separately:

Item	Part Number
ViroSeq HIV-1 Genotyping System User’s Manual	4315267
ViroSeq HIV-1 Genotyping System Quick Reference Guide (available spring 2000)	4315422

Parts Sold Separately: *(continued)*

Item	Part Number
HIV Sample Prep Module	4306027
HIV Sequencing Module, Prt/5´RT	4305611
HIV RNA Control, Prt/5´RT v2	4315236

Modules and Storage Conditions

The HIV-1 Genotyping System comprises four modules. The volumes listed are approximate and exceed volumes needed for 48 reactions.

Sample Prep Module

Tube Name	Volume per Tube (mL)	Color of Tube Cap	No. of Tubes
Viral Lysis Buffer	15	Clear	2
RNA Diluent	2	Clear	3

Store this module at –15 to –25 °C in a manual-defrost freezer that is designated amplicon free.

ViroSeq RT-PCR Module, Prt/5´RT v2

Tube Name	Volume (µL)	Color of Tube Cap	No. of Tubes
AmpliTaq Gold	27.5	Gold	1
RNase Inhibitor	55	White	1
MuLV Reverse Transcriptase	55	Purple	1
HIV PCR Mix v2	1650	Blue	1
HIV RT Mix v2	450	Blue	1
RNA Diluent	2000	Clear	1
AmpErase UNG	55	Green	1
DTT, 100 mM	25	Yellow	1
Low Mass Ladder mix	55	Clear	1
Agarose Gel Loading Buffer	275	Clear	1

Store this module at –15 to –25 °C in a manual-defrost freezer that is designated amplicon free “clean.”

Sequencing Module, Prt/5´RT

Tube Name	Volume (µL)	Color of Tube Cap	No. of Tubes
Formamide	2000	Clear	1
Loading buffer	1000	Clear	1
HIV SEQ MIX A	600	White	1
HIV SEQ MIX B	600	White	1
HIV SEQ MIX C	600	White	1
HIV SEQ MIX D	600	White	1
HIV SEQ MIX F	600	Red	1
HIV SEQ MIX G	600	Red	1
HIV SEQ MIX H	600	Red	1

Store this module at –15 to –25 °C in a manual-defrost freezer that is designated amplicon “dirty.”

RNA Control, Prt/5´RT

Tube Name	Volume (µL)	Color of Tube Cap	No. of Tubes
RNA Control Prt/5´RT v2	50	Clear	1

Store this module at –15 to –25 °C in a manual-defrost freezer that is designated amplicon free “clean.”

Run Module

The run modules used with the Data Collection software are provided on the CD. The names of the run modules are listed below for the different instruments.

Instrument	Run Module
ABI PRISM 310 Genetic Analyzer	HIV-310 Run ModuleH
ABI PRISM 377 DNA Sequencer	HIV Run Module 377-36

IMPORTANT See page 5-4 (ABI PRISM® 310 Genetic Analyzer) or page 6-4 (ABI PRISM® 377 DNA Sequencer) for the directions for installing run modules.

Kit Validation

This RT-PCR procedure has been validated using only the materials specified in this manual. The use of alternative materials and equipment is likely to produce unreliable results. For example:

- MicroAmp tubes are manufactured to have a highly uniform wall thickness, which gives consistent heating and cooling properties. Other tubes may not be manufactured to the same specifications and can lead to poor results.
- The use of thermal cyclers other than those listed can result in considerable variability because of differences in:
  - Temperature ramping times
  - The point at which the timing of each step begins
  - Temperature calibration

Note It is recommended that you regularly calibrate your thermal cyclers.

Note The HIV-1 Genotyping System entitles you to full customer support for the product when you follow this procedure exactly as written. Only the procedure described in this manual has been validated to work correctly.

User-Supplied Materials and Reagents

Preparation of Viral RNA

A list of materials that you must supply for the preparation of viral RNA is given below. These materials should be dedicated to biohazard preparations and not be part of general laboratory equipment.

Description	Supplier
Microcentrifuge tubes, 1.5-mL, sterile and RNase-free	Sarstedt (P/N 72.692.005) or equivalent
Microcentrifuge, refrigerated. Choose one of the following: ♦ 17R, 22R, or 28R Biofuge ♦ GS-15R **Note** The requirements for this centrifuge are that it must be: ♦ Refrigerated ♦ Able to centrifuge samples to 21,000–25,000 *x g*	♦ Baxter /Heraeus ♦ Beckman
Ethanol,[a] 95% nondenatured, ACS[b] Reagent Grade	Major laboratory suppliers (MLS)
Isopropanol (2-propanol), 100% anhydrous	MLS
Pipettes, filter-plugged tips	MLS
Clorox bleach or other EPA[c]-approved disinfectant	This must be purchased through a laboratory supply company and not bought from a general store. **Note** Clorox bleach, purchased through laboratory suppliers, has been specifically validated for use as a disinfectant for work with HIV.
Sterile, deionized, RNase-free water	MLS

a. Store at –20 °C and keep bottles tightly closed to minimize dilution with water vapor in the air.

b. American Chemical Society

c. Environmental Protection Agency

Preparation of PCR Products for Sequencing

A list of materials specific for the preparation of PCR products for sequencing is given below.

Description	Supplier
Sterile, deionized water	MLS
Agarose	Nucleic acid electrophoresis grade
Ethidium bromide	MLS

Sequencing Reactions

A list of materials specific for the sequencing reactions and purification of sequencing ladders is given below. For a list of tube options, see the thermal cycling materials list on page 1-22.

Description	Supplier and Part Number
Sodium acetate, 3 M, pH 4.6	Applied Biosystems (P/N 400320)
Ethanol[a] 95% and 70%, nondenatured, ACS Reagent Grade	MLS
Centri-Sep 96 plates	Princeton Separations (P/N CS-961)
Sterile, deionized water	MLS
Scotch tape 425-3 (for plate precipitation.)	3M Company

a. Store at –20 °C and keep bottles tightly closed to minimize dilution with water vapor in the air.

General Laboratory Equipment

A list of general laboratory equipment needed to perform the assay is given below.

Description	Supplier and Part Number
Centrifuge, benchtop, nonrefrigerated	Eppendorf 5415 or equivalent
Centrifuge, refrigerated, with plate holder if needed	Eppendorf 5403, Jouan CR422/GR-422 or equivalent
RNase free microcentrifuge tubes, 1.5-mL	Sarstedt or MLS
Vortex mixer	MLS
Pipettes, filter-plugged tips	MLS
Conical tubes, 15-mL and 50-mL	MLS

Thermal Cycling

A list of materials that you must supply for thermal cycling is shown below. You will not need to use all of the materials listed because you can choose whether to contain your samples in:

- Individual MicroAmp® Reaction Tubes in a MicroAmp® Base
- MicroAmp® 8-Strip Reaction Tubes in MicroAmp® Trays
- MicroAmp® Optical 96-Well Reaction Plates for the GeneAmp® PCR System

Thermal Cycling Materials

Description	Supplier	Part Number
Thermal cycler GeneAmp® PCR System 9600 or 9700	Applied Biosystems	N801-001 (9600) N805-001 (9700)
MicroAmp Reaction Tubes with Caps, 0.2-mL	Applied Biosystems	N801-0540
MicroAmp 8-Strip Reaction Tubes	Applied Biosystems	N801-0580
MicroAmp® Caps, 8-Strip	Applied Biosystems	N801-0535
MicroAmp® Cap Installing Tool	Applied Biosystems	N801-0438
MicroAmp Base	Applied Biosystems	N801-0531
MicroAmp® 9600 Tray/Retainer Set	Applied Biosystems	N801-5530 403081
MicroAmp Optical 96-Well Reaction Plate	Applied Biosystems	N801-0560
MicroAmp® Full Plate Cover	Applied Biosystems	N801-0550

Sequencing

A list of equipment and consumables that you must supply for sequencing is given below.

Sequencing Equipment and Consumables

Description	Supplier	Part Number
General Materials		
dRhodamine Matrix Standards Kit	Applied Biosystems	403047
Materials Needed If Using the ABI PRISM 310 DNA Sequencer		
310 Capillaries, 61 cm x 50 μm	Applied Biosystems	402840
POP-6™ Performance Optimized Polymer with TSR	Applied Biosystems	402844
Genetic Analyzer Septa for 0.5-mL Sample Tubes	Applied Biosystems	401956
Genetic Analyzer Sample Tubes (0.5-mL)	Applied Biosystems	401957
10X Genetic Analyzer Buffer with EDTA	Applied Biosystems	402824
Genetic Analyzer Retainer Clips	Applied Biosystems	402866
310 Glass Syringe, 1.0-mL	Applied Biosystems	604418
Materials Needed If Using the ABI PRISM 377 DNA Sequencer		
Front Glass Plate, 36-cm	Applied Biosystems	401840
Rear Glass Plate, 36-cm	Applied Biosystems	401839
Step Glass Plate, 36-cm (For 96-well gels only)	Applied Biosystems	4305384
Gel Spacers 36-cm, 0.2 mm thick	Applied Biosystems	401836

Sequencing Equipment and Consumables *(continued)*

Description	Supplier	Part Number
Shark-tooth Combs, 0.2 mm thick, one of the following:	Applied Biosystems	
	36-well	401828
	48-well	402177
	64-well	402180
96-well	0.4 mm mylar sharks tooth	4305385
	0.4 mm single use sharks tooth comb	4309457
TBE, 10X buffer (890 mM Tris, 890 mM boric acid, 20 mM EDTA)	MLS	
Long Ranger™ Singel pack for ABI sequencers 377-36 cm	The FMC Corporation	50696
8-well syringe loader	Kloehn Ltd.	18597
Needles	Kloehn Ltd.	18663

Technical Support

Contacting Technical Support

You can contact Applied Biosystems for technical support by telephone or fax, by e-mail, or through the Internet. You can order Applied Biosystems user documents, MSDSs, certificates of analysis, and other related documents 24 hours a day. In addition, you can download documents in PDF format from the Applied Biosystems Web site (please see the section “To Obtain Documents on Demand” following the telephone information below).

To Contact Technical Support by E-Mail

Contact technical support by e-mail for help in the following product areas:

Product Area	E-mail address
Genetic Analysis (DNA Sequencing)	galab@appliedbiosystems.com
Sequence Detection Systems and PCR	pcrlab@appliedbiosystems.com
Protein Sequencing, Peptide and DNA Synthesis	corelab@appliedbiosystems.com
Biochromatography, PerSeptive DNA, PNA and Peptide Synthesis systems, CytoFluor®, FMAT™, Voyager™, and Mariner™ Mass Spectrometers	tsupport@appliedbiosystems.com
Applied Biosystems/MDS Sciex	api3-support@sciex.com
Chemiluminescence (Tropix)	tropix@appliedbiosystems.com

Hours for Telephone Technical Support

In the United States and Canada, technical support is available at the following times:

Product	Hours
Chemiluminescence	8:30 a.m. to 5:30 p.m. Eastern Time
Framingham support	8:00 a.m. to 6:00 p.m. Eastern Time
All Other Products	5:30 a.m. to 5:00 p.m. Pacific Time

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Switzerland (Rotkreuz)	**41 (0)41 799 7777**	**41 (0)41 790 0676**
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Step	Action
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3	Enter the requested information and your question in the displayed form, then click **Ask Us RIGHT NOW** (blue button with yellow text).
4	Enter the required information in the next form (if you have not already done so), then click **Ask Us RIGHT NOW**. You will receive an e-mail reply to your question from one of our technical experts within 24 to 48 hours.

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Laboratory Guidelines 2

Chapter Overview

Introduction This chapter gives guidelines for the safe and effective use of the ViroSeq™ HIV-1 Genotyping System.

In This Chapter The following topics are covered in this chapter:

General Guidelines

Collecting and Storing Samples

Samples must be collected and stored according to the methods discussed in the DAIDS Virology Manual for HIV Laboratories or other relevant regulations and guidelines (see Appendix C, “Reference.”)

Before You Begin

Before you begin this protocol you must:

- Make sure that your lab has been prepared for work with HIV and is in compliance with all applicable regulations.
- Make sure that all lab personnel know how to work safely with HIV and blood products.
- See the “BIOHAZARD Warning” on page 2-13.
- Know how to protect RNA samples from degradation by RNase enzymes. See Appendix B, “Preventing RNA Degradation.”
- Understand the principles of reverse transcription, PCR, and dye-terminator cycle sequencing.

Sample Handling Protocol

Add reagents to individual tubes or wells in exactly the order given in this manual. Keep only the working sample tube open. All other sample tubes should be capped to restrict sample cross-contamination.

Pay special attention to the stated working temperature. Many reactions are prepared on ice.

IMPORTANT Viral pellets and precipitated RNA are nearly invisible and can be dislodged when the supernatant is removed. Laboratory personnel should read and follow the protocol very carefully at these points in the assay.

Preventing RNA Degradation

RNA is easily degraded by RNases that are present on hands, lab surfaces, and glassware, etc. Take all precautions to prevent RNase contamination of reagents and mixes.

Specifically:

- Store all reagents according to the directions starting on page 1-17.
- Keep all reagents on ice when setting up assays.
- Do not freeze-thaw the RNA control more than four times.
- Always wear gloves when working with RNA.

For more information about handling RNA, see Appendix B, “Preventing RNA Degradation.”

General PCR Practices

PCR is a sensitive technique that can amplify single DNA molecules (Mullis *et al.*, 1987; Saiki *et al.*, 1985; Saiki *et al.*, 1988).

To prevent DNA contamination, which can give failed or false positive results, follow these general guidelines (Kwok and Higuchi, 1989).

- Designate separate areas, equipment, and supplies for:
  - Reagent storage and preparation
  - RNA preparation
  - RT and PCR setup
  - PCR amplification and sequencing
- Wear a new or designated lab coat for each area.
- Change gloves often and before beginning each step.
- Follow a disciplined work flow (i.e., sample prep to RT-PCR to sequencing)
- Once you have worked with open amplicon (post-PCR), do not return to pre-PCR areas until the following day.
- Keep caps on reagents and sample tubes when they are not being used. Minimize arm movements over open tubes during pipetting.
- Regularly clean lab benches and equipment with a solution of 10% bleach.

Background to Laboratory Setup

Sensitivity of PCR

The ViroSeq HIV-1 Genotyping System (and other PCR-based tests) are so sensitive that minute amounts of DNA can be amplified. Precautions must therefore be taken to prevent contamination of samples that have not yet been amplified (Kwok and Higuchi, 1989).

While contamination of amplified DNA with unamplified DNA (genomic DNA) does not pose a problem, ordinary precautions, such as changing pipette tips between samples, should be taken when handling and analyzing PCR product. This action should effectively prevent cross-contamination between samples of amplified DNA.

Sources of Contamination

There are three potential sources of laboratory contamination:

Contamination by Human Genomic DNA from Equipment or the Work Environment

Because of the specificity of the HIV Genotpying System amplification, contamination of samples with non-HIV DNA will not affect results. However, care should be taken while handling and processing samples to prevent chance contamination by human DNA. Gloves should be worn at all times and changed frequently. Sample tubes should be closed when not in use. Dispersal of aerosols should be limited through careful handling of sample tubes and reagents.

Cross-Contamination During Sample Preparation

Extra care should be taken during DNA extraction and RT-PCR setup to prevent transfer of DNA from one sample to another. Use a new, filter-plugged pipette tip for each sample, open tubes carefully, and keep sample tubes closed when you are not using them.

PCR Product Carryover

PCR product carryover occurs when amplified DNA contaminates a sample which has not yet been amplified. It is important to isolate and contain amplified PCR product to prevent it from coming into contact with unamplified samples.

Carryover is a concern because PCR product serves as an ideal template for subsequent amplifications of that same target. A single PCR amplification produces an enormous number of copies (as many

as 10^{13}) that can potentially contaminate samples that have not yet been amplified.

Minute amounts of DNA can be transferred between samples by splashing or the movement of aerosols. As the number of copies of amplified DNA in a completed PCR reaction is so high, inadvertent transfer to one that has not yet been amplified may result in the amplification and detection of the contaminating sequence.

For example, if reuse of a pipette tip transfers 0.1 μL of a completed PCR amplification, then as many as 10^{10} copies of amplifiable sequence will be added to the unamplified sample. By comparison, a nanogram of human genomic DNA contains only about 10^{2} copies of a single-copy locus such as FGA.

UNG AmpErase® UNG (uracil N-glycosylase) is included in the Version 2 kit. It is used during the 50 °C incubation just before AmpliTaq® Gold activation. It destroys the PCR product that is carried over from previous HIV-1 Genotyping System version 2 amplification reactions.

Laboratory Design and Organization

Isolating Work Areas

Special consideration should be given to the design and organization of the laboratory. The laboratory must be organized so that the area where amplified DNA is handled is physically isolated from the work areas for DNA extraction and RT-PCR setup.

Different parts of the HIV Genotyping procedure should be performed in different work areas, each ideally with dedicated equipment and supplies to minimize the likelihood of contamination. The three suggested work areas are:

- Infectious Material Work Area. This work space is for performing the extraction steps.
- RT-PCR Setup Work Area. RT and PCR reagents, and DNA sample additions are made here.
- Amplified DNA Work Area. This area is dedicated to PCR amplification and detection, and other activities that require handling of amplified DNA.

If possible, the Infectious Material Work Area and RT-PCR Setup Work Areas should be separate from each other to prevent potential transfer of exogenous RNA and DNA into the RT-PCR Setup Work Area. If the DNA extraction and RT-PCR Setup Work Areas are in the same room, they should be clearly delineated. Benchtop biological safety cabinets may serve to isolate areas within a room.

The pipettors and other equipment used for RNA extraction are routinely exposed to relatively high concentrations of human genomic DNA and should not be used for RT-PCR setup. Dedicated pipettors and plugged pipette tips should be used for setting up and adding DNA to the PCR reaction tubes.

If possible, use a dedicated area such as a biological safety cabinet with an ultraviolet (UV) source for RT-PCR setup. The UV germicidal lamps in most biological safety cabinets quickly damage any DNA left on exposed surfaces, making it unsuitable for subsequent amplification. All equipment and supplies used for RT-PCR setup should be kept in this cabinet or a dedicated “clean” area at all times. Do not use these items to handle amplified DNA.

Strict physical isolation must be maintained between the area designated for handling amplified DNA and the other areas to avoid transfer of amplified DNA out of the designated work area.

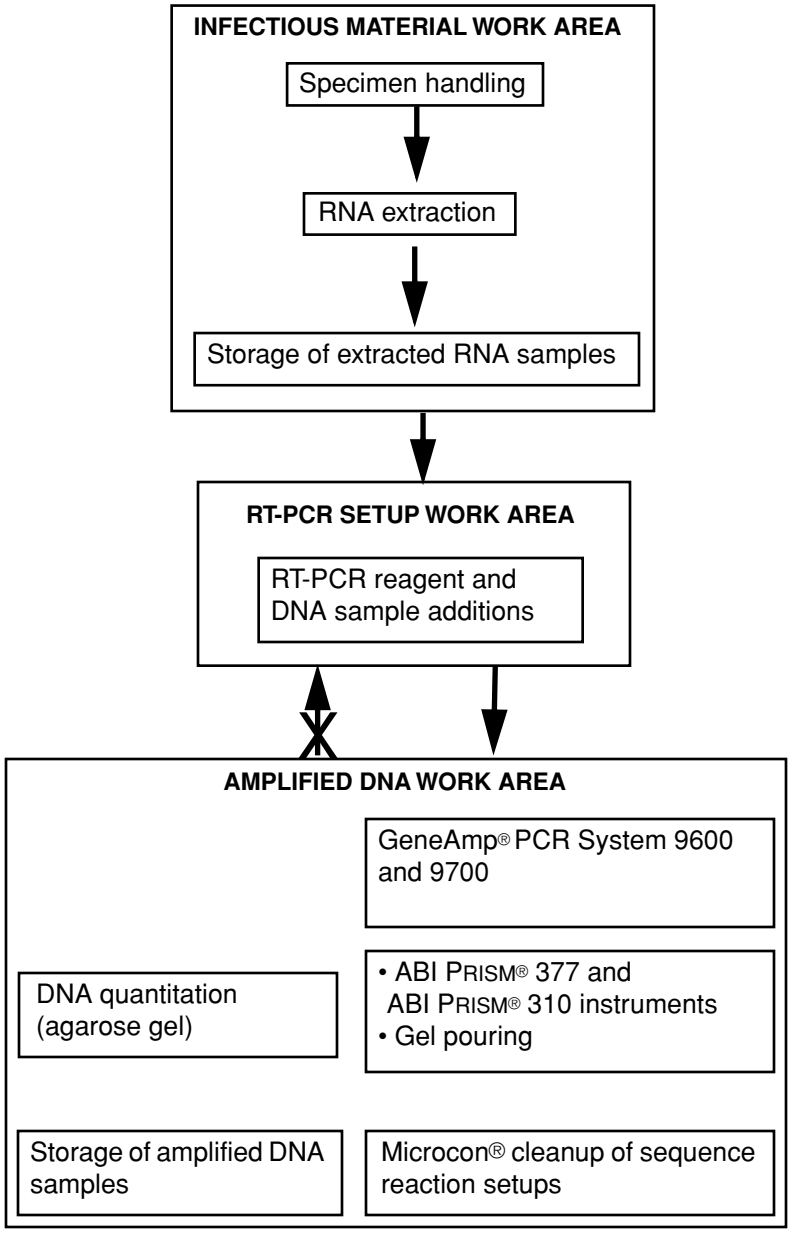
The Amplified DNA Work Area should be in a separate room and must have a dedicated sink. It may make it easier to contain PCR product within this laboratory area if you:

- Use color coded tape to identify the supplies and reagents used for handling PCR product
- Post signs to indicate the use of PCR product in the Amplified DNA Work Area

Because of the equipment used in the Amplified DNA Work Area, a relatively large space is required. This space requirement exceeds the space requirement for RNA extraction and RT-PCR setup. A common mistake is to allocate more space for DNA extraction and RT-PCR setup than for PCR amplification and PCR product detection.

Amplified DNA or equipment and supplies used to handle amplified DNA should not be taken out of the designated work area. If the work area for amplified DNA is in a separate but contiguous room, the user should make sure that air flows toward the amplified DNA area. In addition, it is helpful if there is a separate exit from the Amplified DNA Work Area that does not exit into the pre-PCR work areas.

Work Area Diagram



Infectious Material Work Area

Overview

This work area should be used for:

- Specimen handling
- Extraction of RNA
- Storage of RNA samples

The work surface, equipment, and supplies used in this area must be clean and free of PCR product.

The reagents used for RNA extraction should be prepared in this work area. Portions of the samples to be tested are transferred from this area to the RT-PCR setup Work Area for processing.

Special Precautions

- Limit the number of samples processed at the same time to a manageable number. This precaution will reduce the risk of sample mix-up and the potential for sample-to-sample contamination.
- Use disposable gloves at all times. Change gloves frequently to avoid sample-to-sample contamination. Change gloves whenever they might have been contaminated with DNA and whenever you are leaving the work area.
- Use sterile, disposable, hydrophobic filter-plugged pipette tips and microcentrifuge tubes.

IMPORTANT Do not use gamma irradiation to sterilize microcentrifuge tubes, as this may inhibit PCR subsequently carried out in these tubes.

- Always change pipette tips before handling a different sample.
- Store reagents as small aliquots to minimize the number of times a given tube of reagent is opened. Record the lot numbers of reagents used in each set of samples so that if contamination occurs it can be more readily traced.
- Avoid splashes. Some types of sample tubes have tightly fitting caps which may cause splashing when they are forced open. Centrifuge all liquid to the bottom of the closed tube before opening it. Use a tube-decapper device to open tubes more easily. Clean the tube-decapper device often.
- Include reagent blank controls with each set of DNA extractions to check for the presence of contaminating DNA in the reagents.

- Before setting up the Infectious Material Work Area, clean all work surfaces thoroughly with a 10% (v/v) bleach solution. Use disposable bench paper (for example, Benchkote sheets) on permanent work surfaces to prevent the accumulation of human DNA.
- Wear a labcoat dedicated to pre-amplification sample handling when working in the Infectious Material Work Area.

RT-PCR Setup Work Area

Overview

This work area is used for combining PCR reagents and extracted RNA to the appropriate reaction tubes.

Special Precautions

- Use dedicated pipettors for adding RNA sample to the PCR reaction mixture.
- Use a new sterile, disposable, hydrophobic filter-plugged pipette tip for each RNA sample addition to a PCR reaction tube. Discard used pipette tips.
- To minimize cross-contamination, always add the RNA to the PCR tubes last.
- Make additions to the negative control (no RNA added) tube last. This control will provide a check for contamination occurring during RT-PCR setup.
- Avoid handling the inside surface of the tube caps.
- Change gloves frequently whenever they may have been contaminated with DNA or RNA, or were used to handle anything outside of the RT-PCR setup Work Area.
- Store the RT-PCR reagents in a refrigerator that is located in the Infectious Material Work Area. Do not store the reagents close to samples containing high levels of DNA and RNA.

Amplified DNA Work Area

Overview

This work area should be a physically separate area used only for those activities that involve the handling of amplified DNA. These activities include the:

- Pouring of gels
- Electrophoresis of amplified DNA
- Waste disposal of amplified DNA solutions
- Storage of amplified DNA

Dedicated Equipment and Supplies

Amplified DNA or equipment and supplies used to handle amplified DNA should not be taken out of the Amplified DNA Work Area. Samples that have not yet been amplified should never come into contact with this equipment.

Special Precautions

Even in the Amplified DNA Work Area, amplified DNA should be handled carefully to avoid dispersal around the room. Reducing the dispersal of amplified DNA within this work area will reduce the potential for transfer of amplified DNA to other work areas.

- Always remove your gloves and lab coat when leaving the Amplified DNA Work Area to avoid the transfer of amplified DNA into other work areas.
- Reduce dispersal of DNA around the work area by changing gloves whenever they may have become contaminated with amplified DNA.
- Avoid splashing by opening tubes that contain amplified DNA carefully. It may be helpful to spin down the contents of the amplified DNA tubes before opening. A tube-decapper device makes it easier to open the tubes.
- Use disposable bench paper to cover the work area used to prepare samples for electrophoresis. This prevents the accumulation of amplified DNA on permanent work surfaces. A 10% (v/v) bleach solution should be used periodically to wash exposed work surfaces. Soap and water can also be used to clean work surfaces.
- Use the thermal cycler for the RT-PCR reactions and for the denaturing of the DNA in formamide before sequencing.

- Quantitation of amplified DNA by gel electrophoresis may be performed in this room.
- Do not remove DNA samples from the Amplified DNA Work Area. Store tubes of amplified DNA in this area.

Safety Guidelines

BIOHAZARD Warning

! WARNING ! BIOHAZARD. Biological samples such as tissues and blood have the potential to transmit infectious diseases. Follow the U.S. Department of Health and Human Services guidelines published in *Biosafety in Microbiological and Biomedical Laboratories* (stock no. 017-040-00547-4) and in Occupational Safety and Health Standards, Toxic and Hazardous Substances (29 CFR §1910.1030),or international equivalents, concerning the principles of risk assessment, biological containment, and safe laboratory practices for activities involving clinical specimens. You can obtain additional information by connecting to the government web site, http://www.cdc.gov

Hazardous Waste

! WARNING ! CHEMICAL WASTE HAZARD. Wastes produced by Applied Biosystems instruments are potentially hazardous and can cause injury, illness, or death.

- **Read and understand the material safety data sheets (MSDSs) provided by the manufacturers of the chemicals in the waste container before you store, handle, or dispose of chemical waste.**
- **Handle chemical wastes in a fume hood.**
- **Minimize contact with and inhalation of chemical waste. Wear appropriate personal protective equipment when handling chemicals (e.g., safety glasses, gloves, or clothing).**
- **Seal the waste container with the cap provided after disposing of the contents.**
- **Dispose of the contents of the waste tray and waste bottle in accordance with good laboratory practices and local, state/provincial, or national environmental and health regulations.**

Always use adequate ventilation such as that provided by a fume hood.

Precautions

When working with human blood or blood products, always observe Universal Precautions and follow these general guidelines:

- Never pipette by mouth.
- Do not eat, drink, or smoke in the laboratory.

- Wear two pairs of disposable gloves, sleeve covers on the glove cuffs, a laboratory coat, and eye protection at all times during the procedure. Wear any other personal protective equipment that may be required.
- Use sterile, disposable filter-plugged pipette tips.
- Dispose of unused reagents and waste in accordance with county, federal, state, and local regulations.
- Do not use this kit after its expiration date.
- Read the Material Safety Data Sheets relating to the reagents used in this user's manual.
- Clean and disinfect all work surfaces with 10% (v/v) Clorox bleach or other EPA (Environmental Protection Agency)-approved disinfectant.
- Autoclave any equipment or materials that have come into contact with blood products.
- Do not touch anything outside the hood until you have changed your outer gloves.
- Dispose of all human blood products and associated materials according to your institution's safety guidelines.
- When you finish your work, wash your hands thoroughly with soap and water.

Thermal Cycler Settings 3

Chapter Overview

Introduction

This chapter describes the thermal cycler settings for the GeneAmp® PCR Systems 9600 and 9700. For complete operating instructions, refer to the following manuals:

Operating Instruction for...	Manual
GeneAmp PCR System 9700	
96-Well Sample Base Module	*GeneAmp PCR System 9700 Base Module User's Manual* (P/N 4303481)
96-Well Sample Block Module	*GeneAmp PCR System 96-Well Sample Block Module User's Manual* (P/N 4303480)
GeneAmp PCR System 9600	
	GeneAmp PCR System 9600 User's Manual (P/N 0993-8660)

In This Chapter

The following topics are covered in this chapter:

General Information

Ramping Times

For all thermal cycling profiles, select the shortest ramping times possible.

Reverse Transcription Thermal Cycling

Programming Settings

The following table describes the thermal cycler program for the reverse transcription step using the GeneAmp PCR Systems 9600 and 9700.

Temperature (°C)	Time (min)	Process
42	60	Reverse transcription
99	5	MuLV Reverse Transcriptase denaturation
4	HOLD	Holds for at least 10 min

HIV Amplification Program

GeneAmp PCR System 9600 and 9700

The following table describes the thermal cycling program for the HIV PCR amplification on the GeneAmp PCR Systems 9600 and 9700.

Note Set the 9700 for MaxMode, not 9600 emulation mode.

Number of Cycles	Temperature (°C)	Time	Process
1	50	10 min	AmpErase® UNG contamination control
1	93	12 min	AmpliTaq® Gold activation
40	93	20 sec	DNA denaturation
	64	45 sec	Primer annealing
	66	3 min	Primer extension
1	72	10 min	Final extension
–	4	HOLD	–

HIV Sequencing Program

Programming Settings

The following table describes the thermal cycling program for sequencing the PCR products using the GeneAmp PCR System 9600 and 9700.

Number of Cycles	Temperature (°C)	Time	Process
25	96	10 sec	DNA denaturation
	50	5 sec	Primer annealing
	60	4 min	Primer extension
–	4	HOLD	–

HIV Genotyping Chemistry Protocol 4

Chapter Overview

Introduction This chapter describes the chemistry-related procedures used to prepare samples for sequencing.

In This Chapter The following topics are covered in this chapter:

Planning Your Work

Organizing Your Samples

You have three options for organizing your samples. You can use:

- Individual MicroAmp® Reaction Tubes in a MicroAmp 9600 Tray/Retainer set.
- MicroAmp® 8-Strip Reaction Tubes in MicroAmp® 9600 Tray/Retainer set.
- MicroAmp® Optical 96-Well Reaction Plates.

If you have a small number of samples, it may be more convenient to use individual tubes. Otherwise, use Optical 96-Well Reaction Plates to hold your samples.

Three Procedures Described

There are three procedures for performing sequencing reactions in this manual.

The procedures are for samples contained in:

- Individual MicroAmp Reaction Tubes
- Either MicroAmp Optical 96-Well Reaction Plates or 8-Strip Reaction Tubes in MicroAmp® Trays
- Centri-Sep 96 plates

Use the procedure that is correct for the type of sample container you are using.

Timing

To help you plan your work, the approximate time required for each step in the HIV genotyping procedure is shown below. The actual time will depend on the number of samples you are processing and your familiarity with the procedure.

Times for Each Step:

Step	Approximate Time (hours)
Isolating viral RNA from blood plasma	3–3.5 (for 6–16 samples)
Reverse transcription	1.5
PCR	4.5
Agarose gel electrophoresis	2
Sequencing reactions	3

Times for Each Step: *(continued)*

Step	Approximate Time (hours)
Either: ♦ Ethanol precipitation ♦ Cleanup using the Centri-Sep 96 plate	 1.0 1.5
Sequencing on the ABI PRISM® 377 DNA Sequencer	8
Sequencing on the ABI PRISM® 310 Genetic Analyzer	2 (per sequencing reaction)

Stopping Points

If you are unable to perform the entire procedure without interruption, stop the protocol and store your samples only at certain points.

The acceptable stopping places are:

After you have...	Store at...	See page...
Purified the HIV RNA	–80 ˚C	4-8
Performed the reverse transcription	–15 to –25 ˚C	4-12
Performed the PCR	–15 to –25 ˚C	4-15
Purified the PCR products	–15 to –25 ˚C	4-16
Purified the cycle sequencing reaction products	–15 to –25 ˚C	4-26, or 4-28, or 4-30

You will be reminded in the text when you reach these stopping points.

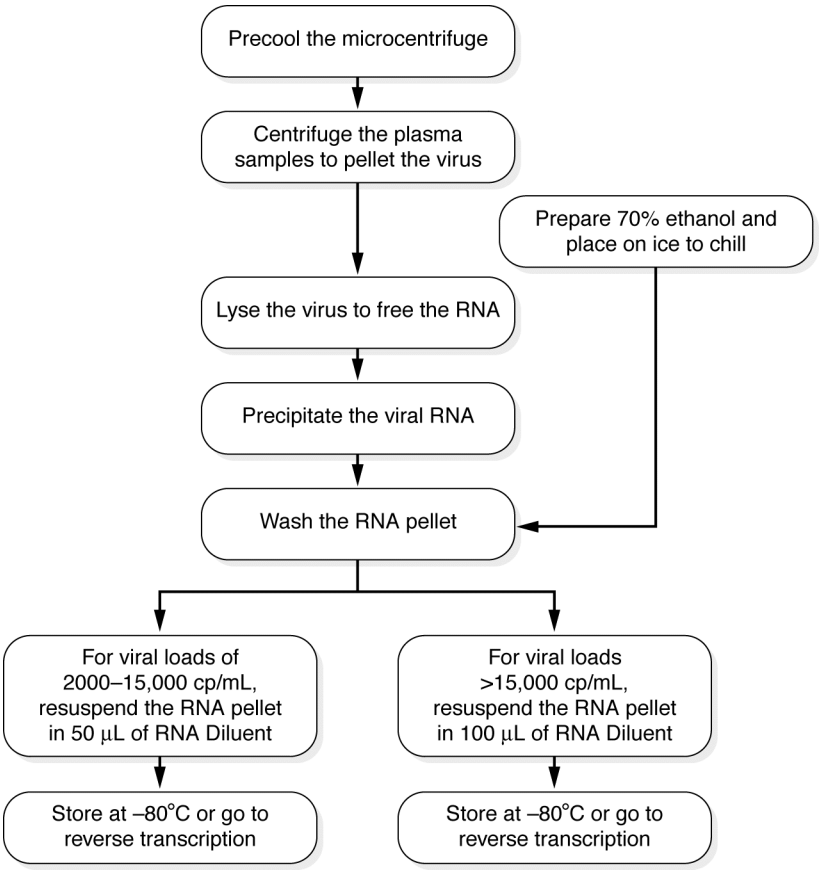
Isolating Viral RNA from Blood Plasma

About the Procedure

The procedure for isolating viral RNA from blood plasma begins with samples of blood plasma with viral load of 2000–750,000 cp/mL. The blood used to prepare the plasma samples must be collected with ACD-A or EDTA anticoagulants. Do not use samples containing heparin.

Flow Diagram

The following diagram summarizes the procedure for isolating and purifying the viral RNA.



Precooling the Centrifuge

To precool a refrigerated, benchtop centrifuge:

Step	Action
1	Install an empty centrifuge rotor according to the manufacturer's instructions.
2	Start a run with the temperature set to 2–6 ˚C.

Pelleting the Virus

The HIV is isolated from each plasma sample by centrifugation. The viruses form a pellet and the plasma supernatant is removed.

To pellet the virus:

Step	Action
1	Thaw the plasma samples.
2	Briefly vortex.
3	Aliquot 0.5 mL of the blood plasma into each Sarstedt screw cap tube and screw on the tube cap.
4	**IMPORTANT** Place an orientation mark on the side of the tube and on the cap.
5	Insert the microcentrifuge tubes into the precooled microcentrifuge rotor with the orientation mark facing the outside rim of the rotor. This will help you locate the pellet after centrifugation.
6	Centrifuge at 4 ˚C at 21,000–25,000 x *g* for 1 hr.
7	During the centrifugation: a. Prepare 70% ethanol and place on ice. b. Thaw the Viral Lysis Buffer by either: – Heating the tubes at 37 ˚C – Leaving the tubes at room temperature until they are thawed c. Briefly vortex the Viral Lysis Buffer tubes and keep them at room temperature until needed. d. Store the RNA diluent on ice until needed. **Note** If you use the room temperature method and a precipitate forms, heat the buffer to 37 ˚C.
8	Remove the tubes as soon as the centrifuge rotor stops.

To pellet the virus: *(continued)*

Step	Action
9	Without disturbing the pellets, which may not be visible, remove the residual supernatants with a disposable fine-tipped transfer pipette. **IMPORTANT** Remove the residual supernatant by pipetting from the side opposite the orientation mark. **! WARNING ! BLOODBORNE/INFECTIOUS WASTE HAZARD. Discard the supernatants following recognized disinfection procedures and in accordance with all local, state, and national bloodborne/infections regulations.**
10	Screw on each tube cap.
11	Increase the temperature in the centrifuge to 15–25 ˚C (room temperature).

Lysing the Viruses

To lyse the viruses:

Step	Action
1	Add 600 µL of Viral Lysis Buffer to each pellet and screw on the tube cap.
2	Vortex each tube gently for 3–5 seconds.
3	Incubate at 15–25 ˚C (room temperature) for 10 minutes.

Precipitating Viral RNA

To precipitate viral RNA:

Step	Action
1	Add 600 µL of 100% isopropanol to each tube, and screw on the tube cap. **! WARNING ! CHEMICAL HAZARD. Isopropyl alcohol is a flammable liquid and vapor. It may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry skin and cause irritation. It may cause central nervous system effects such as drowsiness, dizziness, headache, etc. Please read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.**
2	Vortex each tube gently for 3–5 seconds.
3	Centrifuge samples at room temperature, and 12,500–15,000 *x g* for 15 minutes.

To precipitate viral RNA: *(continued)*

Step	Action
4	Without disturbing the pellets, which may not be visible, remove the supernatants with a fine-tipped transfer pipette. **! WARNING ! BLOODBORNE/INFECTIOUS WASTE HAZARD. Discard the supernatants following recognized disinfection procedures and in accordance with all local, state, and national bloodborne/infections regulations.**

Washing the RNA Pellet

To wash the RNA pellet:

Step	Action
1	Add 1.0 mL of freshly prepared 70% ethanol, precooled to 4 ˚C, to each tube, and then cap. **! WARNING ! CHEMICAL HAZARD. Ethanol is a flammable liquid and vapor. It may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry skin. Exposure may cause central nervous system depression and liver damage. Please read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.**
2	Vortex gently for 3–5 seconds. You do not need to not dislodge the pellet.
3	Replace the microcentrifuge tubes in the rotor with the orientation mark facing the outside rim of the rotor.
4	Centrifuge samples at room temperature and 12,500–15,000 *x g* for 5 minutes.
5	Without disturbing the pellets, which may not be visible, remove the supernatants with a fine-tipped transfer pipette. **! WARNING ! BLOODBORNE/INFECTIOUS WASTE HAZARD. Discard the supernatants following recognized disinfection procedures and in accordance with all local, state, and national bloodborne/infections regulations.**
6	Pulse-spin at low speed for 5–10 seconds to collect the residual ethanol at the bottom of the tube.

To wash the RNA pellet: *(continued)*

Step	Action
7	Remove any residual supernatant with a fine pipette tip (*e.g.,* a Gilson P-200 micropipette tip) or a fine-tipped transfer pipette. The pellets should still be visible. **IMPORTANT** All ethanol must be removed. Any residual ethanol will inhibit the reverse transcription reaction. **! WARNING ! BLOODBORNE/INFECTIOUS WASTE HAZARD. Discard the supernatants following recognized disinfection procedures and in accordance with all local, state, and national bloodborne/infections regulations.**
8	If there is still some residual ethanol in the tubes, leave the tube caps open for 1 minute or longer.

Resuspending the RNA

Use the following procedure to resuspend the washed RNA pellets before storing them.

IMPORTANT From this point forward, unless otherwise specified, all steps must be performed on ice.

To resuspend the RNA:

Step	Action
1	**If...** / **Then...** the viral load is known to be less than 15,000 cp/mL — add 50 µL of RNA Diluent. the viral load is known to be greater than 15,000 cp/mL — add 100 µL of RNA Diluent.
2	Screw on the microcentrifuge tube caps.
3	Resuspend the RNA pellets by vigorously vortexing for 10 seconds.
4	Pulse-spin at low speed for 5–10 seconds to collect the contents at the bottom of the tube.
5	Immediately place on ice.
6	**Either...** / **Or...** proceed immediately to the RT step. See “Reverse Transcription Reactions” on page 4-9. — store the RNA at –60 to –80 ˚C.

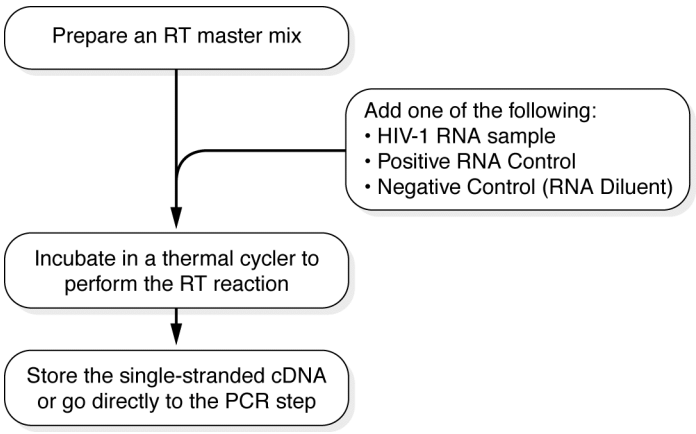
Reverse Transcription Reactions

About the Reaction

During the reverse transcription (RT) reaction, the Moloney murine leukemia virus (MuLV) reverse transcriptase generates a single-stranded cDNA from the HIV-1 RNA.

Overview

To set up the RT reactions, you will perform the following steps:



Preparing the RT Master Mix

To avoid pipetting small volumes, do not prepare single reactions.

IMPORTANT Set up the reactions on ice or on a cold block.

To prepare the RT master mix:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Thaw all reagents and samples and immediately place them on ice.

IMPORTANT Samples and reagents must be kept cold at all times. Do not leave samples and reagents to thaw unattended.</td></tr>
<tr><td>2</td><td>Place the RNase Inhibitor and MuLV Reverse Transciptase on ice.</td></tr>
<tr><td>3</td><td>a. Thaw the HIV RT mix and DTT solution at room temperature.
b. Vortex them briefly to mix.
c. Place them on ice.</td></tr>
<tr><td>4</td><td>Prepare the RT master mix in a RNase-free 1.5-mL microcentrifuge tube, and on ice, as follows:
<table>
<tr><th>Reagent</th><th>Volume for 1 Reaction (µL)</th><th>Volume for 16 Reactions (µL)</th></tr>
<tr><td>HIV RT Mix</td><td>8</td><td>128</td></tr>
<tr><td>RNase Inhibitor</td><td>1</td><td>16</td></tr>
<tr><td>MuLV Reverse Transcriptase</td><td>1</td><td>16</td></tr>
<tr><td>DTT, 100 mM</td><td>0.4</td><td>6.4</td></tr>
<tr><td>Final volume of RT master mix</td><td>10.4</td><td>166.4</td></tr>
</table>
CAUTION CHEMICAL HAZARD. Dithiothrietol (DTT) may cause eye, skin, and respiratory tract irritation, central nervous system depression, and damage to the kidneys. Please read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

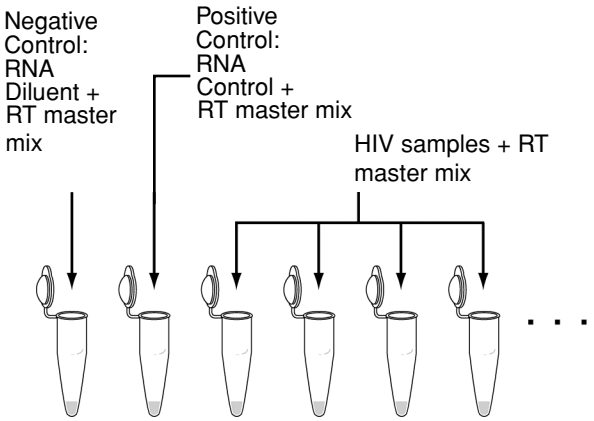
Prepare sufficient volume for 1–2 extra reactions, as some of the mix will be lost during pipetting.</td></tr>
<tr><td>5</td><td>Mix the solution thoroughly by flicking the tube with your fingertip.</td></tr>
<tr><td>6</td><td>Pulse-spin the RT master mix at low speed for 5–10 seconds to collect the contents at the bottom of the tube.</td></tr>
</table>

Preparing the RT Reactions

Use the RT master mix to set up the RT reactions with the viral RNA preps. These reactions should be prepared in precooled 200-µL MicroAmp Reaction Tubes.

IMPORTANT Keep all tubes on ice, or a cold block, while preparing these reactions. Do not freeze and thaw the RNA control more than four times.

To prepare the RT reactions:

Step	Action
1	Add 10 µL of RT master mix to each MicroAmp Reaction Tube. You will need one tube for each HIV sample plus two tubes for the controls.
2	a. Add 10 µL of the negative control, positive control, or viral RNA to each of the MicroAmp Reaction Tubes (from step 1). b. Close the tube cap after each addition.  **Note** The final volume of the reaction mixes is 20 µL.
3	Pulse-spin at low speed for 5–10 seconds to collect the contents at the bottom of the tube. **IMPORTANT** These tubes should be placed on ice until starting the RT reactions.

Performing the RT Reactions

To ensure reproducibility, perform the reverse transcription reactions in MicroAmp Reaction Tubes and use a calibrated thermal cycler for the incubation step.

IMPORTANT Use the correct tray and retainer assemblies for the tubes and thermal cycler.

IMPORTANT The efficiency of the reverse transcription reaction is highly dependent on the conditions of the incubation. Do not vary from the procedure described in the table below.

To perform the RT reaction:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Select the RT program. Review your thermal cycler program for correctness:
<table>
<tr><th>Temperature (°C)</th><th>Time (min)</th><th>Process</th></tr>
<tr><td>42</td><td>60</td><td>Reverse transcribes RNA</td></tr>
<tr><td>99</td><td>5</td><td>Denatures MuLV reverse transcriptase</td></tr>
<tr><td>4</td><td>Hold</td><td>Holds until you are ready</td></tr>
</table></td></tr>
<tr><td>2</td><td>Make sure that the thermal cycler is at 42 ˚C.</td></tr>
<tr><td>3</td><td>Transfer the MicroAmp Reaction Tubes (that are on ice) into the preheated thermal cycler.</td></tr>
<tr><td>4</td><td>Start the thermal cycler.</td></tr>
<tr><td>5</td><td>When the program has finished, either hold the samples at 4 ˚C until you are ready to perform the PCR (for at least 10 minutes) or store at –15 to –25 ˚C.</td></tr>
</table>

Amplifying Viral cDNA by PCR

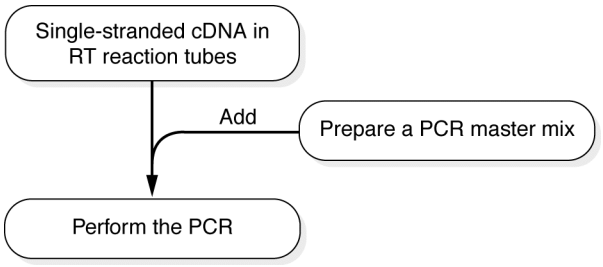
About the PCR Procedure

For convenience and to minimize contamination, PCR is performed in the same tube as the reverse transcription reaction. Using the single-stranded cDNA products of the reverse transcription step as template, the PCR step generates double-stranded DNA suitable for sequencing. The PCR primers in this reaction specifically amplify the HIV protease and the 5´ end of the RT gene. AmpliTaq Gold® is used for PCR because it provides an invisible hot-start, which improves the specificity and efficiency of the reaction.

Note The MuLV reverse transcriptase was inactivated by the previous thermal cycling step in which the samples were incubated at 99 ˚C for 5 minutes.

Overview

You will perform the following steps to amplify your DNA samples:



Preparing the PCR Master Mix

Prepare the PCR master mix as follows:

Step	Action
1	Prepare the PCR master mix by combining the following reagents: **Reagent** / **Volume for One Reaction (µL)** / **Volume for 16 Reactions (µL)** HIV PCR Mix: 29.5 / 472 AmpliTaq Gold: 0.5 / 8 AmpErase® UNG: 1 / 16 **Final volume: 31.0 / 496** **CAUTION CHEMICAL HAZARD. AmpErase uracil n-glycosylase may cause eye and skin irritation. Please read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.** For multiple reactions, prepare sufficient volume for 1–2 extra reactions, as some of the mix will be lost during pipetting.
2	Mix the solution thoroughly by flicking the tube with your fingertip.
3	Pulse-spin the PCR master mix at low speed for 5–10 seconds to collect the contents at the bottom of the tube.

Preparing the PCRs

In this part of the procedure, the PCR master mix is added to each of the RT reaction tubes. Prepare the PCRs as follows:

Step	Action
1	Add 30 µL of PCR master mix to each RT reaction tube, and close the tube cap. The final volume is now 50 µL.
2	Pulse-spin at low speed for 5–10 seconds to collect the contents at the bottom of the tube.

Performing the PCR

Thermal cycling is performed in a GeneAmp® PCR System 9600 or 9700 thermal cycler from Applied Biosystems.

Note The thermal cycling process should take about 4 hours. If it takes 30 minutes less or 30 minutes more, there is probably a problem with the ramping times. In this situation, call a service engineer.

Follow the steps below to amplify the DNA.

Step	Action
1	Place the MicroAmp Reaction Tubes containing the PCRs into the thermal cycler.
2	Select the 9600 or 9700 PCR program shown below. Review your thermal cycler program for correctness.

GeneAmp PCR System 9600 or 9700

Number of Cycles	Temperature (°C)	Time	Process
1	50	10	AmpErase UNG activation
1	93	12 min	AmpliTaq Gold activation
40	93	20 sec	DNA Denaturation
	64	45 sec	Primer Annealing
	66	3 min	Primer Extension
1	72	10 min	Final Extension
–	4	HOLD[a]	–

a. Do not leave tubes on hold for more than 24 hours, because residual UNG activity may destroy your amplified DNA. If the reactions cannot be analyzed within 24 hours, store them at –15 to –25 ˚C.

3	Start the thermal cycler.
4	When the program is complete, either remove the tubes and continue, or store your samples at –15 to –25 ˚C.

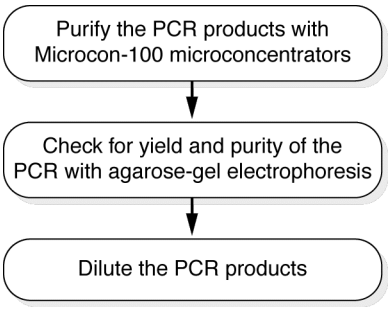
Preparing PCR Products for the Sequencing Reactions

Overview

At this point, the sample tubes contain double-stranded DNA (PCR products) with the 5´ end of the RT gene and the entire protease gene. Before sequencing these PCR products, they must be purified to eliminate unreacted primers and dNTPs.

Purify the PCR products using a Microcon®-100 microconcentrator.

A summary of the preparation steps is shown below.



Concentrating and Purifying the PCR Product

To concentrate and purify the PCR product:

Step	Action
1	Assemble the microconcentrator by inserting a Microcon-100 spin column into one of the supplied 1.5-mL collection tubes. Make sure that the white membrane is face up.
2	Pipette 300 µL of sterile, deionized water onto the top of the microconcentrator. **IMPORTANT** Avoid touching the membrane with the pipette tip.
3	Pipette the entire 50-µL PCR product into the water in the microconcentrator. **IMPORTANT** Avoid touching the membrane with the pipette tip.
4	Seal firmly with the attached tube cap.
5	Centrifuge the prepared microconcentrator in a fixed-angle rotor at room temperature and at 450–550 x *g* for 15 minutes.
6	Open the tube cap and pipette 35 µL of sterile, deionized water into the center of the microconcentrator. **IMPORTANT** Avoid touching the membrane with the pipette tip.

To concentrate and purify the PCR product: *(continued)*

Step	Action
7	a. Remove the microconcentrator from the tube containing filtrate. b. Place the microconcentrator upside down on top of a new 1.5-mL tube. c. Discard the tube with the old filtrate.
8	Centrifuge again at room temperature and at 450–550 x *g* for 5 minutes. This step transfers approximately 30–50 µL of the purified PCR product to the new tube.
9	Remove the microconcentrator and discard it.
10	Close the sample tube, and either place your samples on ice until ready for the next step or store at –15 to –25 ˚C.

Evaluating the PCR Products for Yield and Purity

You may see variability in the yield and purity of the PCR products. This reflects variability in the viral load of the original plasma samples.

To maximize the quality of the sequencing reactions, evaluate the quality of the PCR products before sequencing. To do this, run the PCR products on an agarose gel, and compare them to a “DNA Mass Ladder” containing DNA fragments of known sizes and amounts. Use this information to determine how much to dilute the PCR product before performing sequencing.

Running the Agarose Gel

Follow the procedure below to electrophorese your PCR products.

To run the agarose gel:

Step	Action
1	Prepare: ♦ A 1% agarose gel ♦ Gel buffer containing 0.5 µg/mL of ethidium bromide. **Note CHEMICAL HAZARD. Ethidium bromide is a known mutagen (*i.e.*, it can change genetic material in a living cell and has the potential to cause cancer). Please read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.** **Note** You can either prepare your own gels or purchase prepared gels.
2	For each sample, mix 5 µL of Gel Loading Buffer and 5 µL of purified PCR product.
3	Briefly vortex the tubes.
4	Pulse-spin the tubes at low speed.
5	Load 6 µL of the DNA Mass Ladder solution into lane 1 and 3 µL into lane 2. Pipette accurately because these standards are used as references to determine PCR-product dilutions.
6	Load the entire 10-µL samples onto the remaining gel lanes.
7	Electrophorese at 10 V/cm until the bromphenol blue has migrated at least 5 cm into the gel.
8	Examine the gel under UV light.
9	Photograph your gel using an exposure time that does not saturate the film and shows the differences in intensity of the mass ladder fragments. The correct exposure of the photograph is important, because you will use the information from the gel to determine PCR product dilutions.

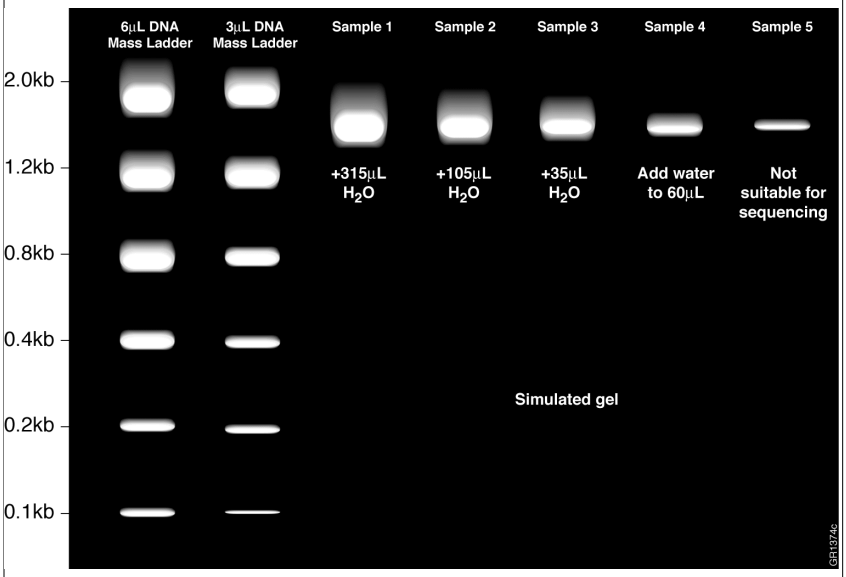
Diluting the PCR Products for Sequencing

The PCR products must be diluted before being sequenced. Follow the table below to determine the correct dilution.

To dilute the PCR products:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Compare the intensity of each PCR-product band to the intensities of the Low Mass Ladder bands. From your comparison, estimate the mass of DNA in the sample. Dilute your sample, which has a volume of 25–45 µL, according to the table immediately below.
<table>
<tr><th>If the mass of the 1.8-kb product band is...</th><th>Then...</th></tr>
<tr><td>greater than 100 ng</td><td>add 315 µL of sterile, deionized water to the sample</td></tr>
<tr><td>between 60–100 ng</td><td>add 105 µL of sterile, deionized water to the sample</td></tr>
<tr><td>between 40–60 ng</td><td>add 35 µL of sterile, deionized water to the sample</td></tr>
<tr><td>between 20–40 ng</td><td>add water to a final volume of 60 µL</td></tr>
<tr><td>less than 20 ng</td><td>the sample may not be suitable for sequencing because there may not be enough DNA</td></tr>
</table>
The table below lists the masses of the DNA fragments in each band of the 3-µL and 6-µL Low Mass Ladders gel lanes.
<table>
<tr><th rowspan="2">Band (kb)</th><th colspan="2">Mass (ng)</th></tr>
<tr><th>6 µL Mass Ladder in a Lane</th><th>3 µL Mass Ladder in a Lane</th></tr>
<tr><td>2.0</td><td>100</td><td>50</td></tr>
<tr><td>1.2</td><td>60</td><td>30</td></tr>
<tr><td>0.8</td><td>40</td><td>20</td></tr>
<tr><td>0.4</td><td>20</td><td>10</td></tr>
<tr><td>0.2</td><td>10</td><td>5</td></tr>
</table>
Note The 0.4 kb and 0.2 kb bands may not be visible on the gel.</td></tr>
</table>

To dilute the PCR products: *(continued)*

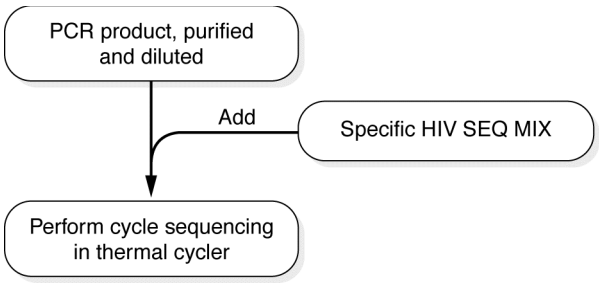
Step	Action
	
2	Vortex briefly.
3	Pulse-spin at low speed for 5–10 seconds to collect the contents at the bottom of the tube. **IMPORTANT** Keep the diluted reactions on ice.

Performing the Cycle Sequencing Reactions

Overview

The sequencing mixes provided with the HIV Genotyping System contain custom sequencing primers formulated with the BigDye™ Terminator sequencing chemistry. The PCR products are used as templates in the cycle sequencing reactions, which generate fluorescently labeled DNA size ladders for sequencing.

A summary of the preparation for cycle sequencing is shown below.



About the HIV SEQ MIXES and Primers

Seven HIV SEQ MIXES are provided, one for each sequence-specific primer.

You have the option of using six primers (A, B, C, F, G, and H) or seven (including D) for your initial sequencing run.

For more information about sequencing primers, see “Primers A and D” on page 1-9.

IMPORTANT Do not expose mixes to light for extended periods.

Preparing Sequencing Reactions

To prepare the sequencing reactions:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Prepare the following sequencing reaction mixes in either:
♦ One MicroAmp Reaction Tube for each HIV SEQ MIX
♦ MicroAmp 8-Strip Reaction Tubes in a MicroAmp Tray
♦ An Optical 96-Well Reaction Plate
<table><tr><th>Component</th><th>Volume for One Reaction (µL)</th></tr><tr><td>Add one of the following HIV SEQ mixes to each tube or plate well:
♦ HIV SEQ MIX A
♦ HIV SEQ MIX B
♦ HIV SEQ MIX C
♦ HIV SEQ MIX D
♦ HIV SEQ MIX F
♦ HIV SEQ MIX G
♦ HIV SEQ MIX H</td><td>12</td></tr><tr><td>Diluted, purified PCR product</td><td>8</td></tr><tr><td>Final Volume</td><td>20</td></tr></table></td></tr>
<tr><td>2</td><td>Close the tube caps or place a MicroAmp® Full Plate Cover over the plate.</td></tr>
<tr><td>3</td><td>Spin at room temperature and at low speed for 5–10 seconds to collect the contents at the bottom of the tube.</td></tr>
<tr><td>4</td><td>Load the MicroAmp tubes, plate, or tray into the thermal cycler.</td></tr>
<tr><td>5</td><td>Select the Cycle Sequencing program. Review your thermal cycler program for correctness:
<table><tr><th>Number of Cycles</th><th>Temperature (°C)</th><th>Time</th></tr><tr><td>25</td><td>96</td><td>10 sec</td></tr><tr><td></td><td>50</td><td>5 sec</td></tr><tr><td></td><td>60</td><td>4 min</td></tr><tr><td>–</td><td>4</td><td>HOLD</td></tr></table></td></tr>
<tr><td>6</td><td>Start the thermal cycler.</td></tr>
</table>

Purifying the Sequencing Reactions in Microcentrifuge Tubes

Overview

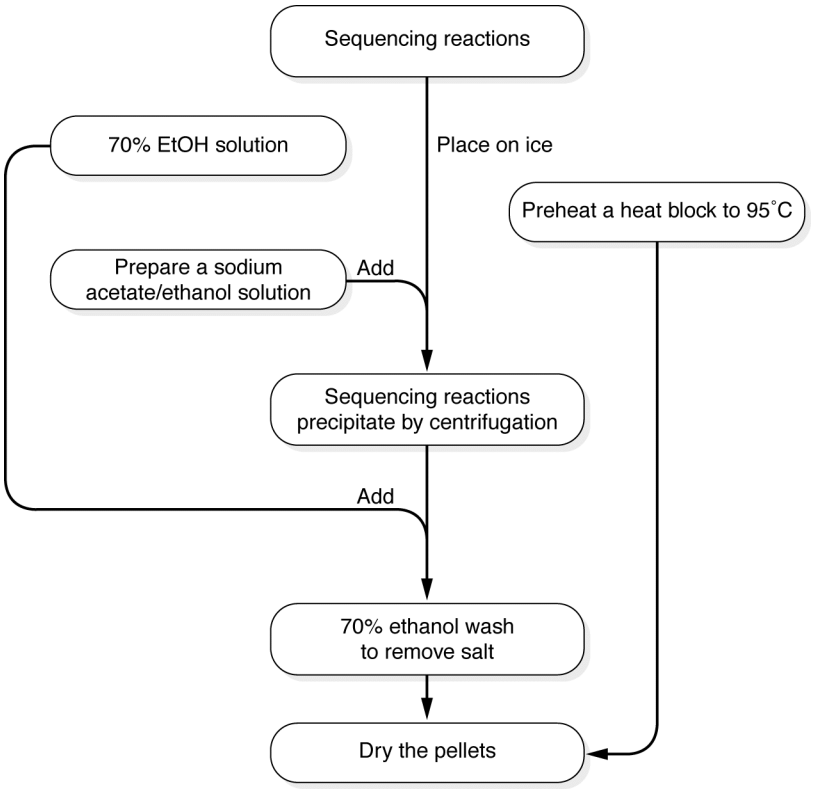
At the end of the cycle sequencing reactions, each tube contains a fluorescently labeled DNA sequence ladder. Before electrophoresis, each sequence must be purified to remove unincorporated dyes and salts, both of which can interfere with the electrophoresis and data analysis.

The sequences can be purified by:

- Ethanol precipitation in individual tubes or 96-well trays, or
- Spin column filtration using Centri-Sep 96 plates

Flow Diagram

The procedure for purifying the sequencing reactions by ethanol precipitation is outlined below.



Ethanol Quality

It is important to use absolute ethanol to precipitate the sequencing reactions. If there is too much water in the ethanol, the precipitation will be less efficient and less DNA will be recovered.

Ethanol Precipitation Choices

You can precipitate the sequencing reactions with ethanol in one of three ways:

- Individual microcentrifuge tubes
- MicroAmp 8-Strip Reaction Tubes in a MicroAmp Tray
- MicroAmp Optical 96-Well Reaction Plates

If you are purifying the sequencing reactions in...	Then...
individual microcentrifuge tubes	follow the protocol in this section.
a MicroAmp Optical 96-Well Reaction Plate or MicroAmp 8-Strip ReactionTubes in a MicroAmp Tray	go to page 4-27.

Purifying the Sequencing Reactions in Tubes

To purify the sequencing reactions in tubes:

Step	Action
1	Label a 1.5-mL microcentrifuge tube for each sample.
2	Begin preheating a heat block that holds 1.5-mL tubes to 95 ˚C.
3	Prepare a sodium acetate/ethanol solution. **IMPORTANT** A fresh batch of this solution must be made for each set of precipitations. **! WARNING ! CHEMICAL HAZARD. Ethanol is a flammable liquid and vapor. It may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry skin. Exposure may cause central nervous system depression and liver damage. Please read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.**

To purify the sequencing reactions in tubes: *(continued)*

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td colspan="2">
<table>
<tr><th>Reagent</th><th>Volume for One Reaction[a] (µL)</th><th>Volume for 13 Plasma Samples using 7 Primers (mL)</th></tr>
<tr><td>3.0 M Sodium acetate, pH 4.6</td><td>2</td><td>0.182</td></tr>
<tr><td>100% Ethanol</td><td>50</td><td>4.550</td></tr>
<tr><td>Final Volume</td><td>52</td><td>4.732</td></tr>
</table>
a. Prepare about 10% more solution than you need.
</td></tr>
<tr><td>4</td><td>Add 52 µL of the sodium acetate/ethanol solution to each labeled 1.5-mL microcentrifuge tube.</td></tr>
<tr><td>5</td><td>Transfer the 20 µL of each sequencing reaction to the corresponding labeled microcentrifuge tube from step 4, and close the tube cap.</td></tr>
<tr><td>6</td><td>Vortex for 3–5 seconds.

IMPORTANT Thorough mixing at this step is essential for efficient precipitation.</td></tr>
<tr><td>7</td><td>Spin at room temperature and maximum speed (greater than 12,500 x g) for 30 minutes.</td></tr>
<tr><td>8</td><td>Remove the sodium acetate/ethanol mixture from each tube by carefully aspirating the solution.</td></tr>
<tr><td>9</td><td>Add 250 µL of cold 70% (v/v) ethanol to the tube, and close the tube cap.</td></tr>
<tr><td>10</td><td>Vortex for 3–5 seconds.</td></tr>
<tr><td>11</td><td>Spin at room temperature and maximum speed (greater than 12,500 x g) for 5 minutes.</td></tr>
<tr><td>12</td><td>Remove all of the ethanol by carefully aspirating the solution.</td></tr>
<tr><td>13</td><td>Dry the pellets in the preheated 95 ˚C heat block for 2 minutes.

IMPORTANT Do not overdry the pellets.</td></tr>
</table>

To purify the sequencing reactions in tubes: *(continued)*

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>14</td><td>
<table>
<tr><th>If you...</th><th>Then...</th></tr>
<tr><td>plan to perform electrophoresis now</td><td>for instructions, see:
♦ Chapter 5, “Using the 310 Genetic Analyzer.”
♦ Chapter 6, “Using the 377 DNA Sequencer.”</td></tr>
<tr><td>do not plan to perform electrophoresis now</td><td>seal the tubes and store at –15 to –25 °C.</td></tr>
</table>
</td></tr>
</table>

Purifying the Sequencing Reactions in 96-Well Plates or Trays

Overview

The procedure for purifying the sequencing reactions in plates or trays is similar to the procedure for purifying the sequencing reactions in MicroAmp Reaction Tubes. The main differences are in the number of samples processed and the centrifugation steps.

Ethanol Quality

It is important to use absolute ethanol to precipitate the sequencing reactions. If there is too much water in the ethanol, the precipitation will be less efficient and less DNA will be recovered.

Purification

To purify the products of the sequencing reactions:

Step	Action
1	Prepare the sodium acetate/ethanol solution. **IMPORTANT** A fresh solution must be made for each set of precipitations. **! WARNING ! CHEMICAL HAZARD. Ethanol is a flammable liquid and vapor. It may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry skin. Exposure may cause central nervous system depression and liver damage. Please read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.**

Reagent	Volume for One Reaction[a] (µL)	Volume for 13 Plasma Samples using 7 Primers (µL)
3.0 M sodium acetate, pH 4.6	2	0.182
100% ethanol	50	4.550
Final Volume	**52**	**4.732**

a. Prepare about 10% more solution than you need.

2	Add 52 µL of the sodium acetate/ethanol solution to each sequencing reaction.
3	Cover the Optical 96-Well Reaction Plate with 3M 425-3 adhesive-backed aluminum foil tape.
4	Seal the tape to the tops of the wells by pressing firmly.

To purify the products of the sequencing reactions: *(continued)*

Step	Action
5	Mix by either inverting three times or vortexing. **IMPORTANT** Thorough mixing at this step is essential for efficient precipitation.
6	Centrifuge at 2000 x *g* for 20 minutes.
7	Remove the foil tape.
8	Invert the plate on a folded lab tissue and centrifuge at 150 x *g* for 1 minute.
9	Add 150 µL of 70% ethanol to each well.
10	Centrifuge at 2000 *x g* for 5 minutes.
11	Invert the plate on a folded lab tissue and centrifuge at 150 x *g* for 1.0–1.5 minutes.
12	If you plan to perform electrophoresis now, see: ♦ Chapter 5, “Using the 310 Genetic Analyzer.” ♦ Chapter 6, “Using the 377 DNA Sequencer.” If you do not plan to perform electrophoresis now, seal the tubes and store at –15 to –25 °C.

Purifying Sequencing Reactions Using a 96-Well Centri-Sep Plate

About Using the Centri-Sep Plate

An alternative method to ethanol precipitation that allows purification of sequencing reactions in a 96-well format is the Centri-Sep 96 Plate (Princeton Separations, PN CS-961). This plate has 96 prepackaged, hydrated spin columns that remove excess dye terminators and nucleotides from the sequencing reactions. Following the spin column centrifugation step, the sequencing reactions are dried in a vacuum centrifuge (speed-vac), dissolved in a loading buffer, and loaded onto the 377 instrument.

Procedure

To purify the sequencing reactions:

Step	Action
1	Allow the Centri-Sep 96 plate to come to room temperature (approximately 2 hours).
2	Remove the adhesive-foil sealing film from the bottom of the Centri-Sep 96 plate and then from the top.
3	a. Place the Centri-Sep 96 plate on top of a MicroAmp Optical 96-Well Reaction Plate, tape the two plates together with a base, and centrifuge at 700 x *g* for 2 minutes. b. Discard the remaining liquid in the Optical 96-Well Reaction Plate by shaking it vigorously. c. Wash and save the MicroAmp Optical 96-Well Reaction Plate for future use as a wash plate.
4	Transfer the sequencing reactions (20 µL) to the individual wells in the Centri-Sep 96 plate. Carefully place samples on the centers of the gel beds. Do not place the samples on the side of the wells. Avoid touching the gel bed with the pipette tips. **Note** Use a multichannel pipettor to transfer the sequencing reactions. This will minimize the chance of sample mix-up during the transfer process.
5	Place a 96-well collection plate (a thin-walled plate provided by Princeton Separations) on a 96-well base, then place the Centri-Sep 96 plate on top.
6	Tape the plates to the 96 well base, making sure that you align the alphanumeric indices on all the plates.

To purify the sequencing reactions: *(continued)*

Step	Action
7	Centrifuge at 700 x *g* for 2 minutes. You should recover approximately 20 µL from each well.
8	Remove the 96-well collection plate.
9	Dry the samples in a speed-vac equipped with the appropriate rotor.
10	Seal the plate with ThermaSeal (included in the Centri-Sep 96 plate package) for storage, at 2 to 8 °C, in a dark box. **Note** The products of sequencing reactions are light sensitive.

Using the 310 Genetic Analyzer 5

Introduction

In This Chapter

This chapter describes the operation of the ABI PRISM® 310 Genetic Analyzer used its the corresponding autosampler tray.

The following topics are covered in this chapter.

Assumptions

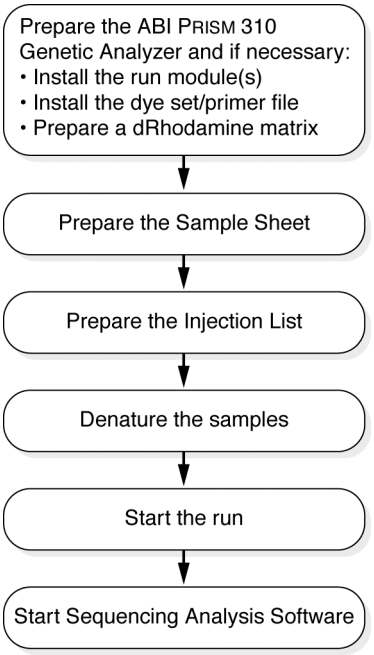
The instructions in this chapter assume that you are already familiar with the ABI PRISM 310 instrument.

For more detailed information about how to perform a sequencing run, refer to the *ABI PRISM 310 Genetic Analyzer User's Manual* (P/N 903565).

Overview of the Procedure

At This Stage Following ethanol precipitation, the samples are dye-labeled sequence ladders that are ready to be run on an automated DNA sequencer. The samples may be run in 1.5-mL microcentrifuge tubes, MicroAmp® 8-Strip Reaction Tubes, or MicroAmp® Optical 96-Well Reaction Plates.

Flow Diagram An overview of a sequencing run is shown below.



Preparing the Instrument

For First Time Users

If you are using the HIV Genotyping System for the first time you:

- Will need to install the appropriate run modules.
- May need to:
  - Make a dRhodamine matrix file
  - Install the appropriate dye set/primer (mobility) file.

Run Modules

A run module is a file that is used to define the run conditions during data collection on the 310 Genetic Analyzer. Run conditions include electrophoresis voltage, temperature, and run time.

Use the run module named HIV-310 Run ModuleH that is included on the CD-ROM provided with the HIV Genotyping System.

Do not use the other run modules on this CD-ROM because they define run conditions that are inappropriate for the current kit configuration.

About the Dye Set/Primer File

A dye set/primer file is used by the ABI PRISM® DNA Sequencing Analysis software to compensate for the mobility effects that different dyes impart on the fragments of the sequence ladder during electrophoresis. Use of the proper dye set/primer file will result in more evenly spaced peaks in the electropherograms.

For the sequences generated with the HIV Genotyping System, use the dye set/primer file named DT POP- 6{BD Set-Any Primer}.

Obtaining the Dye Set/Primer File

If you do not already have the dye set/primer file named DT POP- 6{BD Set-Any Primer}, you can obtain it from:

- The Applied Biosystems Product Software Library web site at: http://www.appliedbiosystems.com/techsupport
- Your local Field Applications Specialist. Call Applied Biosystems Technical Support or your local sales office for more information

IMPORTANT Dye set/primer file names for the BigDye™ terminators are similar to those for the dRhodamine terminators. If you inadvertently select a dye set/primer file for the dRhodamine terminators, it is possible to reanalyze the data with the correct file.

Installing the Run Module and Dye Set/Primer File

To install the run modules and dye set/primer file:

Step	Action
1	Copy the run module (HIV-310 RunModuleH) into the **Module** folder located within the **Collection software** folder of your computer.
2	If necessary, copy the DT POP6{BD Set-Any Primer} dye set/primer file for the ABI PRISM 310 Genetic Analyzer into the ABI folder within the System Folder.
3	Restart the Data Collection software and/or DNA Sequencing Analysis software if either was open while the files were installed.

Preparing an Instrument/Matrix File

How Instrument/Matrix Files Are Used

Instrument/matrix files are used by the ABI PRISM DNA Sequencing Analysis software to compensate for the overlapping fluorescence emission spectra of the different dyes in a dye set.

Before Running Your Samples

The following table lists the actions to take before running your samples and explains where to get additional information.

If...	Then...
you have not already generated a matrix file on the ABI PRISM 310 Genetic Analyzer using the dRhodamine Matrix Standards Kit, the HIV-310 RunModuleH, and POP-6™ polymer	you will need to do this when you run your first samples.
For more information about...	**Refer to...**
making the matrix file	♦ The ABI PRISM 310 *Genetic Analyzer User's Manual* (P/N 903565) and ♦ The *Automated DNA Sequencing Chemistry Guide* (P/N 4305080)

Note Although BigDye terminators and dRhodamine terminators require different dye set/primer files, both chemistries use a matrix made with the dRhodamine matrix standards.

Note Matrix files are specific to a particular instrument, polymer, and dye set.

Preparing the Instrument

To prepare the ABI PRISM 310 Genetic Analyzer:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Prepare 1X Genetic Analysis Buffer by mixing:
<table>
<tr><th>Reagent</th><th>Volume (mL)</th></tr>
<tr><td>ABI PRISM® 310 Genetic Analyzer Buffer with EDTA</td><td>1.5</td></tr>
<tr><td>Deionized water</td><td>13.5</td></tr>
<tr><td>Final Volume</td><td>15.0</td></tr>
</table></td></tr>
<tr><td>2</td><td>If switching applications, or if the sequencing polymer has not been used for more than one week, discard the polymer and flush water through the pump block.</td></tr>
<tr><td>3</td><td>Inspect and clean the detector window.</td></tr>
<tr><td>4</td><td>Install the 61-cm capillary.</td></tr>
<tr><td>5</td><td>Select Autosampler Calibration from the Instrument menu of the Data Collection software.

Follow the prompts to calibrate the autosampler.</td></tr>
<tr><td>6</td><td>Verify that there are no visible crystals in the POP-6 polymer.

If you do see crystals, leave the polymer at room temperature until they are completely dissolved.

CAUTION CHEMICAL HAZARD. POP-6 polymer may cause eye, skin and respiratory tract irritation. Please read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Use for R&D purposes only.</td></tr>
<tr><td>7</td><td>Take up approximately 0.5 mL of POP-6 into the 1-mL glass syringe.</td></tr>
<tr><td>8</td><td>Install the 1-mL glass syringe on the pump block.</td></tr>
<tr><td>9</td><td>Prime the block with polymer.</td></tr>
<tr><td>10</td><td>Load 1X Genetic Analysis Buffer into the buffer reservoir.</td></tr>
<tr><td>11</td><td>Load the autosampler with the following reagents:
<table>
<tr><th>Reagent</th><th>Autosampler Position</th></tr>
<tr><td>1X Genetic Analysis Buffer</td><td>1</td></tr>
<tr><td>Water (in the buffer vial)</td><td>2</td></tr>
<tr><td>Water (in a 1.5-mL microcentrifuge tube with the cap removed)</td><td>3</td></tr>
</table></td></tr>
</table>

Preparing the Sample Sheet and Injection List

Preparing the Sample Sheet

To prepare the sample sheet:

Step	Action
1	From the **File** menu, select **New**.
2	Select the sequence sample sheet that corresponds to the autosampler tray that you are using (either 48 or 96 well). The **Sample Sheet** window opens.
3	Enter the names of your samples into the **Sample Name** column using the naming convention: **sample ID-visit date-lab code-[primer-analysis date**. For more information about sample naming conventions, see page1-14.
4	In the **Dye Set/Primer** list, select **DT POP6{BD Set-Any Primer}.**
5	a. Click the **Dyeset/Primer** column heading to select the entire column. b. From the **Edit** menu, select **Fill Down** (Cmd-D).
6	In the Matrix list, select the appropriate matrix file. For more information, see page 5-4.
7	From the **File** menu, select **Save**.
8	Enter a name for your sample sheet.
9	Save your sample sheet in the **Sample Sheet** folder within the **310 Collection Software** folder.
10	Click **Save**.

Preparing the Injection List

To prepare the injection list:

Step	Action
1	From the **File** menu, select **New**.
2	Select **Sequence Injection List**. The **Injection List** dialog box opens.
3	a. Click the **Sample Sheet** field to display a pop-up menu of sample sheets stored in the **Sample Sheet** folder. b. Select the sample sheet you want to use for the run. The injection list is automatically filled with information from the selected sample sheet.
4	Select the **HIV-310 RunModuleH** from the **Module** pop-up menu.
5	The blank row inserted at the bottom of the injection list must remain blank. If you need to add a blank row, highlight the last row of the injection list and select **Clear** from the **Edit** menu. All items are cleared from the row.
6	Set the capillary read length to 50 cm.

Denaturing the Samples and Starting the Run

Denaturing the Dye-Labeled DNA Fragments

Before loading the samples, they must be dissolved in Template Suppression Reagent (TSR) and denatured.

To denature the samples:

Step	Action
1	Add 20µL of TSR to each sample pellet. **IMPORTANT** Only prepare the number of samples that can be run within 48 hours. Do not leave samples in TSR for longer than 48 hours.
2	Close the tubes, or cover the wells.
3	Vortex for 3–5 seconds.
4	Pulse-spin at room temperature for 5–10 seconds to collect the contents at the bottom of the tube.
5	Heat the samples in a thermal cycler at 95 °C for 2 minutes.
6	Transfer each denatured sequencing reaction to a 0.5-mL Genetic Analyzer Sample Tube.
7	Place a Genetic Analyzer Septum on each Genetic Analyzer Sample Tube.
8	Load the autosampler tray with the Genetic Analyzer Sample Tubes.
9	Click **Start** in the **Injection List**. The run will begin after the heat plate has reached 50 °C.

Analyzing the Sequencing Data

DNA Sequencing Analysis Software Settings

The following table lists the settings to use in the Sample Manager window of the ABI PRISM DNA Sequencing Analysis software:

Sample Manager	Choose
Basecaller	ABI-CE1
Basecaller Settings	HIV 580 See “Creating a New Basecaller Setting” below.
Dye Set/Primer file	DT POP6{BD Set-Any Primer}
Instrument file	The file you have generated on the ABI PRISM 310 Genetic Analyzer, using the dRhodamine Matrix Standards Kit, the HIV-310 Run ModuleH, and POP-6 polymer.

For additional instructions, see “Analyzing the Sequencing Data” on page 6-13.

Creating a New Basecaller Setting

About Basecaller Settings

The Basecaller setting in DNA Sequencing Analysis software allows you to define automatically the number of bases that you want to process in your sequence data files.

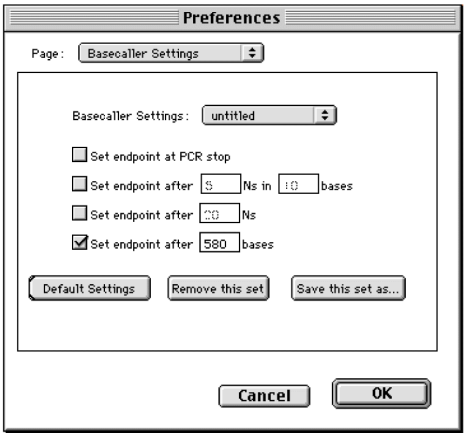
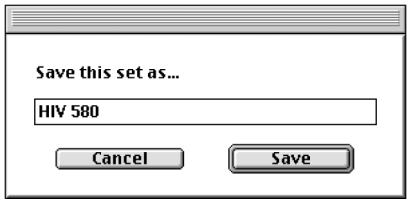
For the data files generated with the ViroSeq™ HIV-1 Genotyping System, all sequences should be processed to stop at 580 bases.

Procedure

To create a new Basecaller Setting:

Step	Action
1	Start the DNA Sequencing Analysis software.
2	Select **Preferences** from the **Edit** menu.
3	Select **Basecaller Settings**. The **Preferences** dialog box opens.
4	Click the **Create a set** button. The title of the button changes to **Save this set as**.

To create a new Basecaller Setting: *(continued)*

Step	Action
5	Select the checkbox labeled **Set endpoint after *n* bases**, and enter **580** in the text box. The **Basecaller Settings** should now look like this: 
6	Click the **Save this set as** button. The following dialog box opens: 
7	Enter **`HIV 580`** in the text box.
8	Click **Save**.

Using the 377 DNA Sequencer 6

Chapter Overview

Introduction This chapter describes the operation of the ABI PRISM® 377 DNA Sequencer used with its corresponding autosampler tray.

In This Chapter The following topics are covered in this chapter:

Assumptions The instructions in this chapter assume that you are already familiar with the ABI PRISM 377 DNA Sequencer.

For more information about performing a sequencing run, see the ABI PRISM 377 *DNA Sequencer User's Manual* (P/N 4303613).

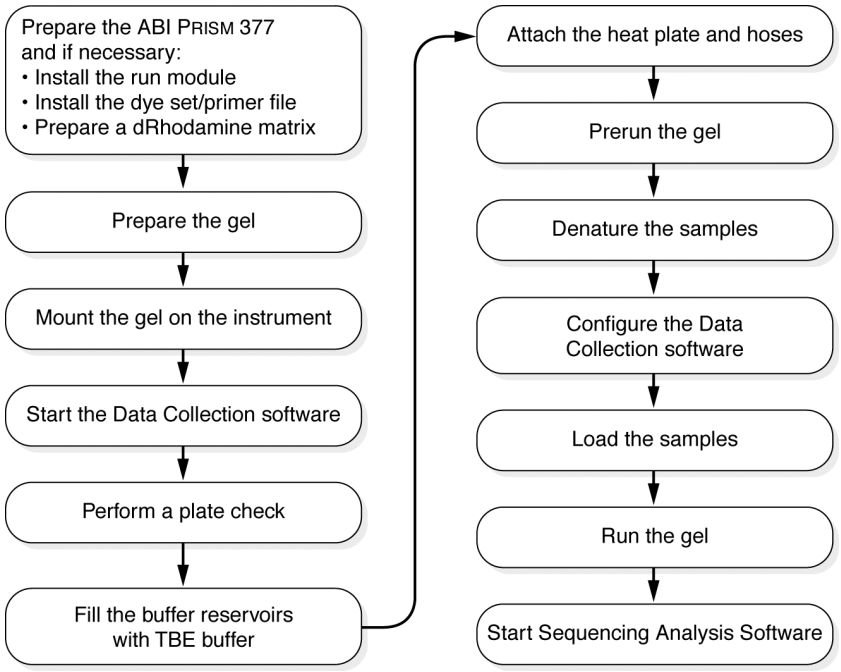
Overview of the Procedure

At This Stage

Following ethanol precipitation, the samples are ready to be run on an automated DNA sequencer. The sequences may be in 1.5-mL microcentrifuge tubes, MicroAmp® 8-Strip Reaction Tubes, or MicroAmp® Optical 96-Well Reaction Plates.

Flow Diagram

An overview of the procedure used to complete the sequencing is shown below.



Preparing the Instrument

For First Time Users

If you are using the HIV Genotyping System for the first time you:

- Will need to install the appropriate run modules.
- May need to:
  - Make a dRhodamine matrix file
  - Install the appropriate dye set/primer (mobility) file.

Run Modules

A run module is a file that is used to define the run conditions during data collection on the 377 DNA Sequencer. Run conditions include electrophoresis voltage, temperature, and run time.

Use the run module named HIV Run Module 377-36 that is included on the CD-ROM provided with the HIV Genotyping System. This module is used for all gel formats on the 377 DNA Sequencer.

Do not use the other run modules on this CD-ROM because they define run conditions that are inappropriate for the current kit configuration.

About the Dye Set/Primer File

A dye set/primer file is used by the DNA Sequencing Analysis software to compensate for the mobility effects that different dyes impart on the fragments of the sequence ladder during electrophoresis. Use of the proper dye set/primer file will result in more evenly spaced peaks in the electropherograms.

For the sequences generated with the HIV Genotyping System, use the dye set/primer file named DT {BD Set-Any Primer}.

Obtaining the Dye Set/Primer File

If you do not already have the dye set/primer file named DT {BD Set-Any Primer}, you can obtain it from:

- The Applied Biosystems Product Software Library web site at: http://www.appliedbiosystems.com/techsupport
- Your local Field Applications Specialist. Call Applied Biosystems Technical Support or your local sales office for more information

IMPORTANT Dye set/primer file names for the BigDye™ terminators are similar to those for the dRhodamine terminators.

If you inadvertently select a dye set/primer file for the dRhodamine terminators, it is possible to reprocess the data with the correct file.

Installing the Run Modules and Dye Set/Primer File

To install the run modules and Dye Set/Primer file:

Step	Action
1	Copy the HIV Run Module 377-36 into the **Module** folder within the **Collection software** folder. The run module is on the CD-ROM supplied with the HIV Genotyping System.
2	If necessary, copy the DT{BD Set Any Primer} file for your instrument into the **ABI** folder within the **System** folder.
3	Restart the Data Collection software and/or DNA Sequencing Analysis software if either was open when the files were installed.

Preparing an Instrument/Matrix File

How Instrument/Matrix Files Are Used

Instrument/matrix files are used by the ABI PRISM DNA Sequencing Analysis software to compensate for the overlapping fluorescence emission spectra of the different dyes in a dye set.

Preparing an Instrument/Matrix File

The following table lists the actions to take before running your samples and explains where to get additional information.

If...	Then...
you have not already generated an instrument file for use with the dRhodamine Matrix Standards Kit, and an HIV Run Module 377-36 on the ABI PRISM® 377 DNA Sequencer	you will need to do this while running your first samples.
For more information about...	**Refer to...**
generating an instrument file	directions in the following: ♦ The *ABI PRISM dRhodamine Matrix Standards Kit User Bulletin* (P/N 904917), or ♦ The ABI PRISM 377 *DNA Sequencer User's Manual* (P/N 4303613)

Note Although BigDye terminators and dRhodamine terminators require different Dye Set/Primer files, both chemistries use the same instrument/matrix file made with the dRhodamine matrix standards.

Note The instrument/matrix file that you generate is specific to a particular instrument and dye set. You cannot use an instrument/matrix file that has been created on a different instrument or with a different dye set.

Preparing the Gel

Follow the directions in the Long Ranger™ Singel pack for ABI PRISM sequencers 377-36 cm to prepare the gel for 36, 48, and 64 lanes. Instructions for preparing a 96-lane gel are on page 6-11. For information on plate, spacer, and comb information, see "Sequencing" on page 1-23.

! DANGER ! CHEMICAL HAZARD. Long Ranger Singel pack contains ammonium persulfate, acrylamide, formaldehyde and urea. In addition, the Long Ranger Singel pack may contain one or more crosslinkers that have various toxicological effects. Contact FMC for further information about the composition of your Lot. Ammonium persulfate is an oxidizer, and contact with other materials may cause a fire. Exposure causes burns to the eyes, skin, and respiratory tract. Acrylamide is harmful if in contact with the skin or if swallowed. It may cause eye, skin and respiratory tract irritation. Exposure to acrylamide may cause damage to the nervous system and reproductive system. Acrylamide may also cause an allergic reaction, is a possible cancer and birth defect hazard, and may cause kidney damage. Formaldehyde may cause an allergic reaction and is a probable human cancer hazard. Urea may cause eye, skin and respiratory tract irritation. Please read the Long Ranger Singel pack MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Filling Out the Sample Sheet

To fill out the sample sheet:

Step	Action
1	Start the Data Collection software.
2	From the **File** menu, select **New**. A **Create New** message box opens.
3	Click **Sequence Sample**.
4	Enter the names of your samples into the **Sample Name** column using the naming convention: **sample ID-visit date-lab code-[primer-analysis date** For more information about sample naming conventions, see page1-14.
5	In the **Dye Set/Primer** pop-up menu, select **DT {BDSet AnyPrimer}**.
6	Select the appropriate instrument/matrix file in the **Instrument File** pop-up menu.

To fill out the sample sheet: *(continued)*

Step	Action
7	Fill down the **Dye Set**/**Primer** and **Instrument File** columns by clicking the column headings and selecting the **Fill Down** command from the **Edit** menu.
8	Enter additional information into the **Project** and **Comment** columns if desired.
9	Save the sample sheet into the **Sample Sheet** folder.

Performing a Plate Check

Perform a plate check to make sure that the plates are clean.

To perform the plate check:

Step	Action	
1	Mount the gel in the instrument.	
2	Click **Plate Check**.	
3	Examine the baseline data.	
	If...	**Then...**
	each of the four-color baselines is flat	continue with the procedure.
	one or more of the baselines are noisy	remove the plates from the instrument, clean them, and repeat the plate check. **IMPORTANT** Do not use the gel.

Setting Up a Run

To set up a run:

Step	Action
1	From the **File** menu, select **New**.
2	Click **Sequence Run**. This opens the **Run** window.
3	Select **Plate Check E** from the **Plate Check Module** list.
4	Select **Seq PR 36E-1200** from the **Prerun Module** list.
5	Select **HIV Run Module 377-36** from the **Run Module** list.

To set up a run: *(continued)*

Step	Action
6	From the **Lanes** list, select the correct number of lanes for the gel you are running (that is, 24, 36, and so forth). **Note** You must select the number of lanes before selecting the sample sheet.
7	Select the correct comb type for the gel you are using.
8	a. Click the **Sample Sheet** field to display a pop-up menu of sample sheets stored in the **Sample Sheet** folder. b. Select the sample sheet for the current run. The **Run File** is automatically filled with information from the selected sample sheet.
9	From the **Instrument File** list, select the instrument file that was made with the dRhodamine Matrix Standards Kit on the instrument you are running.
10	In the **Well-to-Read Distance** box, make sure that **36 cm** is selected.

Prerunning the 36-, 48-, and 64-lane Gel

To prerun the 36-, 48-, and 64-lane gel:

Step	Action
1	Add 1X TBE buffer to both lower and upper buffer chambers (about 1500-mL total).
2	Use a syringe and needle to rinse 1X TBE buffer around the comb, removing air bubbles.
3	Attach the heat plate and hoses.
4	Close the instrument door.
5	Click **Prerun**. The **Scan** window is displayed.
6	Prerun the gel for approximately 20 minutes or until the temperature reaches 50 °C. **Note** While prerunning the gel, you can denature your samples.

Denaturing the Samples

Denaturing the Sequencing Reactions

Before loading your samples, they must be dissolved in a gel-loading buffer solution and denatured. We recommend preparing an excess of formamide loading buffer solution. The volumes in the following table will provide sufficient excess solution for the indicated number of reactions.

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Prepare the following loading buffer/formamide solution in a 1.5-mL microcentrifuge tube. You need 5 µL for each sequencing reaction.

Prepare a fresh mix for each use.

<table>
<tr><th>Chemical</th><th colspan="5">Volumes for 1, 36, 48, 64, and 96 Reactions (µL)</th></tr>
<tr><td></td><td>1</td><td>36</td><td>48</td><td>64</td><td>96</td></tr>
<tr><td>Formamide</td><td>5</td><td>190</td><td>250</td><td>330</td><td>500</td></tr>
<tr><td>Loading buffer</td><td>1</td><td>38</td><td>50</td><td>66</td><td>100</td></tr>
<tr><td>Final Volume</td><td>6</td><td>228</td><td>300</td><td>396</td><td>600</td></tr>
</table>

! WARNING ! CHEMICAL HAZARD. Formamide is harmful if absorbed through the skin and may cause irritation to the eyes, skin, and respiratory tract. It may cause damage to the central nervous system and the male and female reproductive system, and is a possible birth defect hazard. Please read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.</td></tr>
<tr><td>2</td><td>Resuspend each sample pellet in 5 µL of the above solution, and vortex for 3–5 seconds.</td></tr>
<tr><td>3</td><td>Pulse-spin at room temperature and low speed for 5–10 seconds.</td></tr>
<tr><td>4</td><td>Heat the samples in a thermal cycler at 95 °C for 2 minutes.</td></tr>
</table>

Loading the Gel and Starting the Run for 36, 48, and 64 Lanes

Loading Samples

To load samples onto the gel:

Step	Action
1	Pause the prerun. This allows the instrument to continue heating while the samples are loaded.
2	Load the samples into alternate lanes, for example, lanes 1, 3, 5, 7.... Use the table below to select a sample volume to load.

Comb Size	Sample Loading Volume (µL)
36	1.5
48	1.0
64	0.8

Step	Action
3	Prerun samples into the gel for 2 minutes.
4	Pause the electrophoresis prerun.
5	Load the remaining samples into the remaining alternate lanes, for example, lanes 2, 4, 6, 8....

Starting the Run

To start the run:

Step	Action
1	In the **Run** window, click **Cancel**.
2	Click **Terminate**. This ends the prerun. **Note** It is very important to terminate the prerun. Data will not be collected in the prerun mode.
3	Click **Run**.
4	Name the gel file.

To start the run: *(continued)*

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>5</td><td>Click Save to save the gel file into your run folder.

The run takes 7 hours.

The Status window should show the following setpoints:

<table>
<tr><th>Setpoint</th><th>Value</th></tr>
<tr><td>EP Voltage</td><td>1680 V</td></tr>
<tr><td>EP Current</td><td>50 μA</td></tr>
<tr><td>EP Power</td><td>150 W</td></tr>
<tr><td>Gel Temperature</td><td>51 °C</td></tr>
<tr><td>Laser Power</td><td>40 mW</td></tr>
</table>
</td></tr>
</table>

Running Samples on a 96-Lane Gel

About Running Samples on a 96-Lane Gel

Running samples on a 96-lane gel involves some protocol modifications as outlined in the procedure below. The plates are prepared using special stepped front plates, and the upper buffer chamber contains water, not 1X TBE during the prerun step. These modifications help to improve sequence quality when the samples are loaded. Once the gel is loaded and prerun for 2 minutes, 10X TBE is added to the upper buffer chamber to produce 1X TBE.

Sequencing Reaction Methods

To prepare the sequencing reactions for the 96-lane sequencing gel, use either of the following methods:

Method	See Page
Purifying the Sequencing Reactions in 96-Well Plates or Trays	4-27
Purifying Sequencing Reactions Using a 96-Well Centri-Sep Plate	4-29

Procedure

To run samples on a 96-lane gel:

Step	Action
1	Prepare the sequencing gel with the stepped front glass plate and using the smooth/flat side of a 96-lane sharks-tooth comb.
2	After the gel is polymerized, place it on the ABI PRISM 377 instrument.
3	Prepare a 1X TBE buffer solution in the lower buffer chamber.
4	Add 540 mL of deionized water to the upper buffer chamber. **Note** Running the samples into the gel with water in the upper chamber will improve the sequence resolution.
5	Load 1 μL of each sample onto the gel using a multichannel loader. **Note** If you are not loading all 96 lanes of the gel, make sure that you add loading buffer/formamide mix to the empty wells. Loading buffer/formamide mix helps to minimize differences in salt concentration between lanes and improves the lane tracking after electrophoresis. It also results in straighter tracking on the outside lanes of the gel. **Note** Using an 8-channel gel loader helps to load 96 samples. A Kloehn loader is recommended.

To run samples on a 96-lane gel: *(continued)*

Step	Action
6	Prerun the samples onto the gel for 2 minutes.
7	End the prerun.
8	Remove the comb from the gel and rinse the surface of the gel with a syringe.
9	Add 60 mL of 10X TBE to the upper buffer chamber, and mix completely.

Analyzing the Sequencing Data

Steps Before Processing Data

Before processing the data with the DNA Sequencing Analysis software, perform the following steps for each sequence:

Step	Action
1	Check the tracking for each lane on the gel image.
2	<table><tr><th>If the lane tracking is...</th><th>Then...</th></tr><tr><td>acceptable</td><td>proceed using the DNA Sequencing Analysis software</td></tr><tr><td>not acceptable</td><td>retrack the lane. Refer to the *DNA Sequencing Analysis Software User's Manual*.</td></tr></table>
3	Extract data from all retracked lanes, then proceed with the DNA Sequencing Analysis software.

Sequencing Analysis Settings

The following table lists the settings to use in the Sample Manager window. Once the settings have been verified, click the Start button. After analysis, check the data for each sequence to determine if the start point is appropriately set.

Parameter	Select...
Basecaller	ABI100
Basecaller Settings	HIV 580 See "Creating a New Basecaller Setting" below.
Dye Set/Primer file	DT{BD Set Any Primer}
Instrument file	The file you have generated on the ABI PRISM 377, using the dRhodamine Matrix Standards Kit and the HIV Run Module 377-36.

Creating a New Basecaller Setting

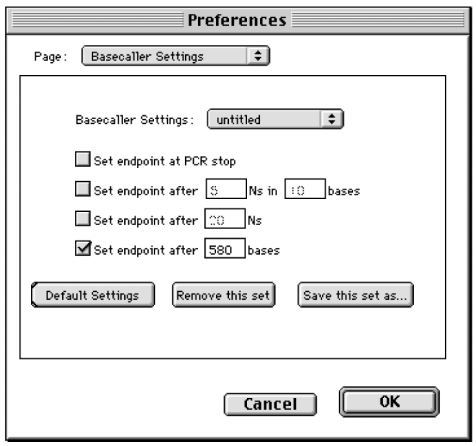
About Basecaller Settings

The Basecaller setting in the DNA Sequencing Analysis software allows you to define automatically the number of bases that you want to process in your sequence data files.

For the data files generated with the ViroSeq™ HIV Genotyping System, all sequences must be processed to stop at 580 bases.

Procedure

To create a new Basecaller Setting:

Step	Action
1	Start the DNA Sequencing Analysis software, and select **Preferences** from the **Edit** menu and **Basecaller Settings** from the submenu. The **Preferences** dialog box opens.
2	Click the **Create a set** button. The title of the button changes to **Save this set as**.
3	Select the checkbox labeled **Set endpoint after *n* bases** and enter **580** in the text box. The **Basecaller Settings** should now look like this: 

To create a new Basecaller Setting: *(continued)*

Step	Action
4	Click the **Save this set as** button. The following dialog box opens: 
5	Enter **`HIV 580`** in the text box, and click **Save**.

Analyzing HIV Sequencing Data 7

Introduction

In This Chapter

This chapter describes how to use the ViroSeq™ HIV-1 Genotyping System Software to perform genotyping and mutation analysis of HIV-1.

IMPORTANT The sample files must be correctly processed using the ABI PRISM® DNA Sequencing Analysis software before the HIV-1 Genotyping System Software can be used.

The following topics are covered in this chapter:

Installing the Software

Introduction

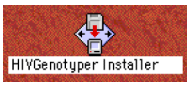
The HIV-1 Genotyping System Software is supplied on the CD provided with the HIV Genotyping System Starter Kit.

Before Installing the Software

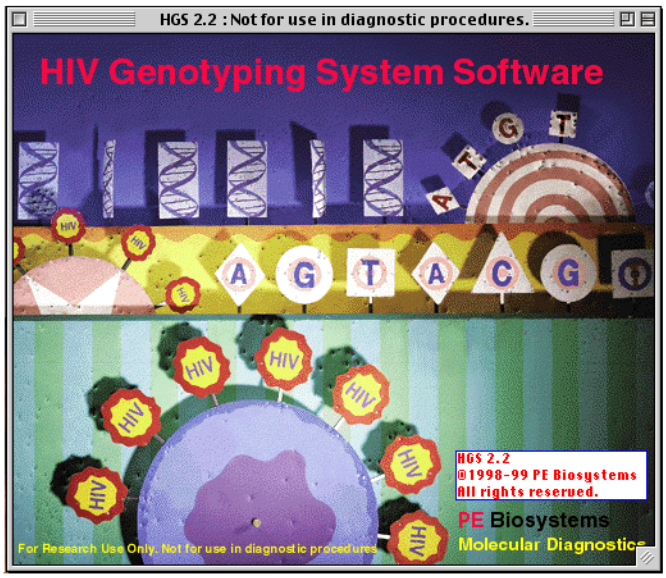
Make sure that your computer meets the minimum requirements for running the software. For more information about computer requirements, see page 1-15.

Procedure

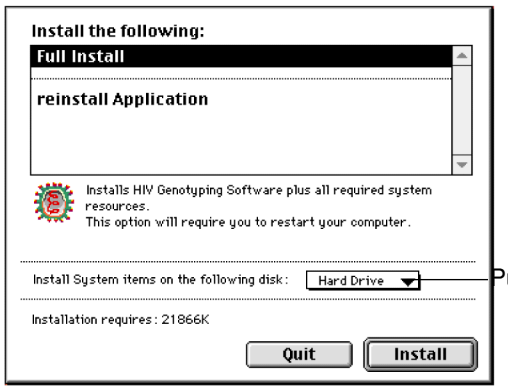
To install the HIV-1 Genotyping System Software:

Step	Action
Turning Off Extensions and Disabling Virus Protection	
1	Close all applications.
2	Turn off all extensions in the Macintosh® Control Panels Extensions Manager, except for the Apple® CD-ROM extension. If you turn off the CD-ROM extension, you will not be able to use the CD.
3	Disable any virus protection software.
4	Restart the computer to register the changes to the **Extensions Manager**.
Installing the Software	
1	Insert the CD into the CD drive. The icon for the CD will appear on the desktop.
2	Double-click the icon for the CD.
3	Double-click **HIV Genotyper Installer**.  A startup display appears.

To install the HIV-1 Genotyping System Software: *(continued)*

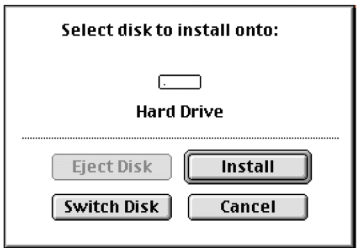
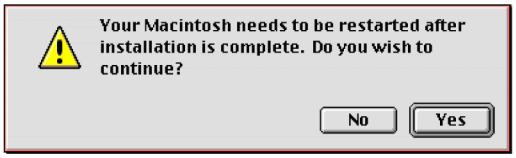
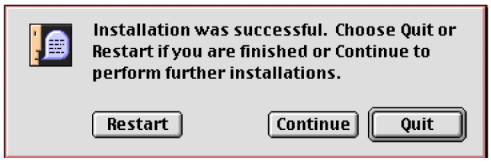
<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td></td><td> 
</td></tr>
<tr><td>4</td><td>Click Continue.

The following message box appears:

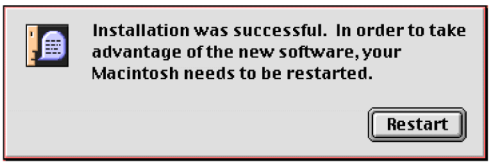
 

Pull-down menu</td></tr>
<tr><td>5</td><td><table>
<tr><th>If you...</th><th>Then select...</th></tr>
<tr><td>are installing the software for the first time</td><td>Full Install.</td></tr>
<tr><td>reinstalling the software</td><td>Reinstall Application.</td></tr>
</table></td></tr>
</table>

To install the HIV-1 Genotyping System Software: *(continued)*

Step	Action
6	Use the pull-down menu to select the disk onto which the program will be installed. You will usually select the name that is assigned to your hard drive icon in the Finder, for example, Hard Drive. 
7	Click **Install**. The following dialog box opens: 
8	Click **Install**. An alert box will remind you that after installation the computer will have to be restarted. At this time you have the option to cancel the installation. 
9	Click **Yes**. If the installation is successful, the following message box opens: 

To install the HIV-1 Genotyping System Software: *(continued)*

Step	Action
10	Click **Restart, Continue**, or **Quit.** The following dialog box opens: 
11	a. Click **Restart**. b. Turn on the extensions that you want to use in the Macintosh computer Control Panels Extensions Manager. c. Restart the computer a second time to register the changes to the Extensions Manager.

Using the ViroSeq HIV-1 Genotyping System Software

Software Overview

Genotype analysis of the sequencing data is performed using the HIV-1 Genotyping System Software. This software processes the six or seven sample files that correspond to a single plasma sample to generate a project. A project is an assembly of the sample files containing all the sequencing information required to produce a genotyping result. The project format supports manual review and editing of the electropherogram data to generate a final consensus sequence for the HIV protease and RT genes.

Sources of Information

The HIV-1 Genotyping System Software uses three sources of information in the analysis process. The following table describes where these information-containing files are located.

File	Where located
Segments created from the DNA Sequencing Analysis files.	In the folder selected when creating the files.
pNL4-3 reference sequence, GenBank Accession Number M19921.	within the software as part of the gene profile
Compendium of known resistance mutations from the Los Alamos Database (see step 6 on page 7-9).	

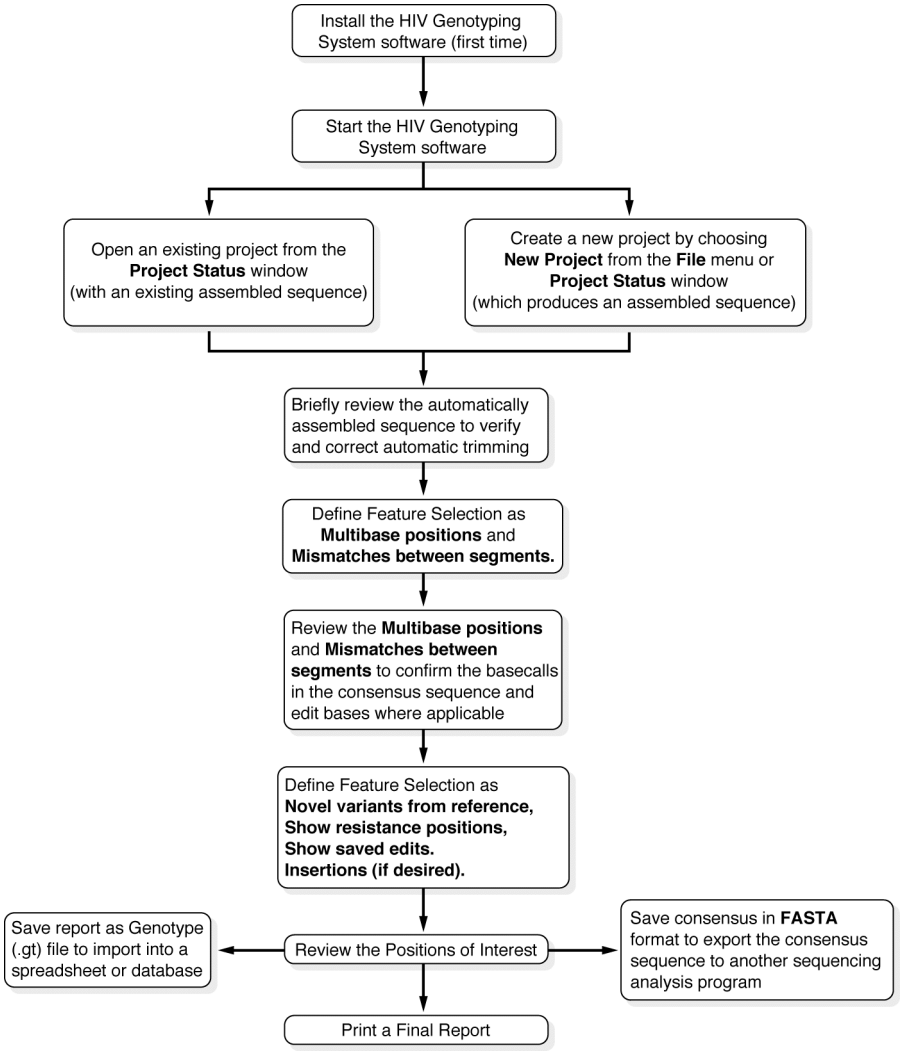
For More Information

For more information about using the HIV-1 Genotyping System Software, the following have been included:

Item	See Page
Flow Diagram	7-7
Automated Processes	7-8
Tutorial: Using the HIV-1 Genotyping System Software	7-10

Flow Diagram

The following flow diagram shows the software steps involved in the genotyping process:



Automated Processes

The results are stored in the project file.

Automated software processing sequence:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>The sample data files are imported into the HIV-1 Genotyping System Software.

The relevant segment information are automatically extracted from a folder that you select and are used to create the project file.

The sample name is used to group the files into projects. All titles with identical sample names will be grouped for analysis

Note The sample file name is not used to group the projects.

<table>
<tr><th>If...</th><th>Then...</th></tr>
<tr><td>you want to include comments unique to a particular sample file (e.g,. the primer name)</td><td>use the open square bracket ([) to separate this information from the sample name.

Note Any information to the right of the open bracket is ignored.</td></tr>
<tr><td>For more information about...</td><td>See page...</td></tr>
<tr><td>Sample naming</td><td>1-14</td></tr>
</table>
</td></tr>
<tr><td>2</td><td>After the project file is created, the segment electropherogram data are automatically analyzed for mixed base positions and the appropriate International Union of Biochemists (IUB) letter codes are assigned.

See Appendix E, “IUB Codes.”

A position is defined as a mixed position if in one segment the smaller peak is at least 30% of the larger peak, and a second segment shows the presence of the smaller peak.</td></tr>
<tr><td>3</td><td>The segments are compared to the reference sequence pNL4-3 to determine segment identity.

Based on the segment identity, regions of poor or unnecessary sequences are automatically inactivated or trimmed.

The sequence in a trimmed region plays no further role in the analysis. It is, however, available for viewing.</td></tr>
</table>

Automated software processing sequence: *(continued)*

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>4</td><td>The trimmed segments are assembled and a project consensus sequence is derived using the CAP assembly algorithm (Huang, 1992).

Positions that disagree between two overlapping segments are categorized as Mismatches. Mismatches can be either individual basecall differences as well as insertions and deletions (indels).</td></tr>
<tr><td>5</td><td>The consensus is compared to the pNL4-3 reference sequence.

Positions within the consensus sequence that disagree with the reference sequence are categorized as variants (see step 6 below for further discussion of variants).</td></tr>
<tr><td>6</td><td>The consensus is compared to the table of known HIV antiviral resistance mutations (Korber et al., 1997).
<table>
<tr><th>Consensus positions that vary from the reference and...</th><th>Are categorized as...</th></tr>
<tr><td>match the table of known HIV antiviral resistant mutations</td><td>Reported Variant</td></tr>
<tr><td>are not included in the table</td><td>Novel Variant</td></tr>
</table></td></tr>
<tr><td>7</td><td>Any position along the consensus in which at least one of the segments has a multibase position is categorized as a “Multibase Position.” The smaller peak height must be at least 30% of the height of the larger peak.
<table>
<tr><th>If...</th><th>Then...</th></tr>
<tr><td>this multibase position results in one or more amino acids for this codon</td><td>each is listed by the single letter amino acid code in the translation.</td></tr>
</table></td></tr>
</table>

Tutorial: Using the HIV-1 Genotyping System Software

Steps to Using the Software

To use the HIV-1 Genotyping System Software:

Step	Action
1	From the **Apple** menu, select **About This Computer**. Make sure that there is a minimum of 32 MB of memory available by closing nonessential applications.
2	Open the HIV-1 Genotyping System Software by double-clicking the icon for HIV Analysis. The HIV-1 Genotyping System Software startup display appears, followed by the **Project Status** window. The **Project Status** window can be empty, or it may contain a list of projects that are currently in the **Completed** folder.
3	In the **Project Status** window, click the **New** button to begin a new project. If you want to open a project that has already been analyzed but is not in the **Completed** folder: a. Click the **Find** button. The **Macintosh computer navigation** window opens. b. Scroll to the location of the project and click **Open**.
4	a. Locate the QA10 set of data files in the **QA10** folder. The **QA10** folder is located within the **Demo** folder in the **Projects** folder of the **HIV Genotyping** folder. b. Click **Open** when you see the list of sequence data files. A progress box appears, showing how the HIV-1 Genotyping System Software is processing the set of data files. When processing is complete, the **Navigation** window opens.
5	Click the blue turn triangle to view the entire **Navigation** window with the **Alignment** View of the segment assembly.  For more information, see “Reviewing the Assembled Sequence” on page 7-20.

To use the HIV-1 Genotyping System Software: *(continued)*

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>6</td><td>Click on the left end of the Navigation bar to open the View/Edit window.

This presents the first position of interest in the sequence for the 5´ end of the alignment (primers A, D, and F).

You can take the following actions:
<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>a.</td><td>To select the positions of interest that you want to examine in your initial review of the data
a. Click the Feature Selection button in the View/Edit window.
The Feature Selection window appears.
b. Select the checkboxes labeled Multibase Positions and Mismatches Between Segments.
For more information, see “Setting Active Positions of Interest” on page 7-34.</td></tr>
<tr><td>b.</td><td>Close the Feature Selection window by clicking the close box. The selection is automatically saved.

The change in the number of positions of interest will be apparent on the Navigation Bar (i.e., the number of colored vertical lines will decrease).</td></tr>
<tr><td>c.</td><td>Click the Auto jump on edits button so that as you make the edits, the cursor moves automatically to the next position of interest to the right.
<table>
<tr><th>If...</th><th>Then...</th></tr>
<tr><td>you do not edit a position of interest</td><td>click the right arrow button to move to the next position of interest.</td></tr>
</table></td></tr>
</table></td></tr>
</table>

To use the HIV-1 Genotyping System Software: *(continued)*

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>7</td><td>Continue editing until all currently selected positions of interest have been reviewed.

You can take the following actions:
<table><tr><th>To...</th><th>Then...</th></tr><tr><td>make edits</td><td>use letters on the keyboard or use the buttons on the Editing Palette (see “Editing Bases” on page 7-35).</td></tr><tr><td>perform any trimming or untrimming that is necessary</td><td>see “Trimming Sequence Segments” on page 7-33.</td></tr></table></td></tr>
<tr><td>8</td><td>When you are satisfied with your edits, you can take the following actions:
<table><tr><th>To...</th><th>Then...</th></tr><tr><td>save the project</td><td>select Save from the File menu.</td></tr><tr><td>save the project in the FASTA or .GT format</td><td>select Save Fasta (⌘-F) or Save Genotype (⌘-G) from the File menu.

For more information, see “Saving Projects” on page 7-40.</td></tr></table></td></tr>
<tr><td>9</td><td>Review the automated processing and edits.

To review the automated processing and your manual edits to this point, select History from the Window menu. The History window appears.

For more information, see “The History Window” on page 7-26.</td></tr>
</table>

To use the HIV-1 Genotyping System Software: *(continued)*

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>10</td><td>To review the electropherogram or make additional edits.
<table>
<tr><th>If...</th><th>Then...</th></tr>
<tr><td>you want to review the electropherograms</td><td>go to the Feature Selection window, and select the features that you would like to inspect.

For more information, see “Setting Active Positions of Interest” on page 7-34.</td></tr>
<tr><td>you make additional changes</td><td>save them as in step 8 on page 7-12.</td></tr>
</table></td></tr>
<tr><td>11</td><td>From the File menu, select Print (⌘-P) to print a report.

For more information, see “Printing a Report” on page 7-41.</td></tr>
</table>

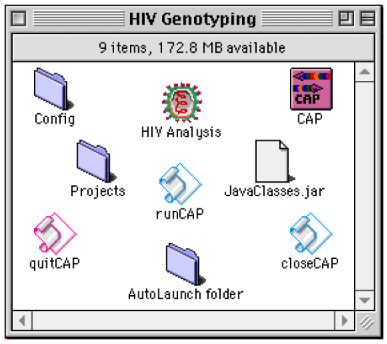
HIV Genotyping Folder Organization

Introduction

During installation, the HIV-1 Genotyping System Software installer creates folders that are used to store your data before, during, and after analysis.

Folder Diagram

The following diagram shows the contents of the HIV Genotyping folder:



HIV Genotyping Folder Contents

The following table describes the items in the HIV Genotyping folder:

Contents of the HIV Genotyping Folder

This folder...	Contains...
Config	Resources that are used by the software.
Project	The following items:

Folder	Description
Demo	Contains data for demonstrating this software.
Completed	Contains completed project files. Also contains the Reports folder, where completed Genotype (.gt) and FASTA (.fasta) reports are placed.
Archives folder	Initially this folder is empty. Move the files that you want to save for future reference into this folder.

Contents of the HIV Genotyping Folder *(continued)*

This folder...	Contains...
Do Not Modify the Following Items	
AutoLaunch	AutoLaunch application. Use to set the day and the folder in which the HIV-1 Genotyping System Software polls the DNA Sequencing Analysis software for sample files (see "Setting Up the Application" on page 7-44).
quitCAP, runCAP, and closeCAP Scripts	Utility files that are used by the software to perform sequence assembly.
Cap	Sequence assembly software module.
JavaClasses.jar	All the classes of executable software.
HIV Analysis	Application software.

About Projects and Sequence Segments

What Is a Project?

A project is the organization and assembly of the six or seven sequence segments that are generated from a single RT-PCR product. The project will also contain all user-defined edits and changes to the sequence data.

All the necessary data for each segment is included in the appropriate project. Segments belonging to a project are identified by a unique sample name. The name of the project is the same as the sample name.

How Sequence Segments are Defined

The sequence segments that are contained in a project are defined by the:

- Folder in which the sample files are stored.
- Sample name that you assigned the segments when filling out the sample sheet of the Data Collection software.

  For more information on sample naming, see page 1-14.

Starting the Software

Before Starting the Software

Ensure that there is sufficient Finder memory by closing all nonessential applications.

To Check the Amount of Memory Available

From the Apple menu, select About This Computer. Make sure that at least 32 MB of memory is available.

Creating Projects Manually

The following table describes how to create projects manually:

To...	Then...
manually create individual projects	place into one folder all the data files for the sample that you wish to assemble. **Note** Commonly, these files will already be part of a run folder.

If sample files for more than one project are included in a folder, projects will be sorted according to the sample name.

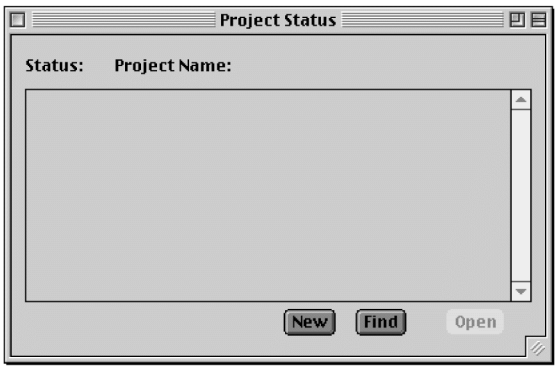
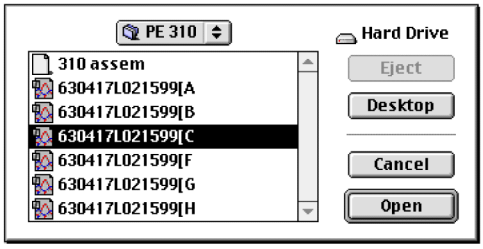
Procedure

To start the HIV-1 Genotyping System Software:

Step	Action
1	Double-click **HIV Analysis.** This opens the HIV-1 Genotyping System Software, and the **Project Status** window appears.
2	<table><tr><th>If you want to...</th><th>Then see page...</th></tr><tr><td>Create a new project</td><td>7-17</td></tr><tr><td>Open an existing project</td><td>7-18</td></tr></table>

Creating a New Project

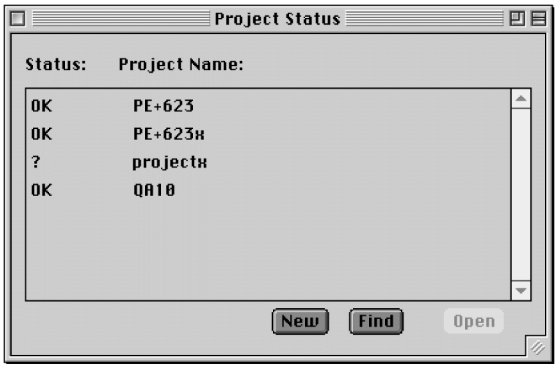
Procedure To create a new project:

Step	Action
1	Click **New** in the **Project Status** window.  This opens the standard Macintosh computer navigation box. 
2	Navigate to the folder that contains the samples you want to use to create a project.
3	Select any file in the folder, and click **Open**. The HIV-1 Genotyping System Software creates the projects from all the sample files that are in the folder, assembles and analyzes them and displays the data.

Opening a Previously Created Project

Where Projects Are Saved

The software saves previously created projects in the Completed folder. All the projects that are in this folder are listed in the Project Status window.



The following table describes the values for the Status field:

If Status is...	Then...
OK	the segments were found at the expected locations.
?	one or more input segments were not identified by the software.

Procedure

The following table describes two ways to open a project:

To open projects...	Take this action	
listed in the **Project Status** window	**You can either...**	**Result**
	a. Select the project that you want to open. b. Click **Open**.	The **Navigation** window opens.
	Double-click the project name.	

To open projects...	Take this action
present in other folders	a. In the **Project Status** window, select **Find**. This opens a standard Macintosh computer navigation box. b. Navigate to the folder that contains the project that you want to open. c. Select the project, and click **Open**.

Reviewing the Assembled Sequence

About This Step

Whether you have created a new project or opened an existing project, the next step in the genotyping procedure is to review the assembled sequence.

Reviewing Process

The following table defines the steps in your reviewing process:

Step	Action
1	Use the features in the **Navigation** window to review the entire assembled project.
2	You will use the **View Edit** window to trim poor quality data, edit the assembled sequence, and generate a consensus sequence. The **Navigation** and **View Edit** windows are linked, so that a change in analysis settings results in corresponding changes in the **Navigation** window.

The Navigation Window

About the Navigation Window

The Navigation window provides an overview of the overlap in the sequence segments and the number of positions of interest.

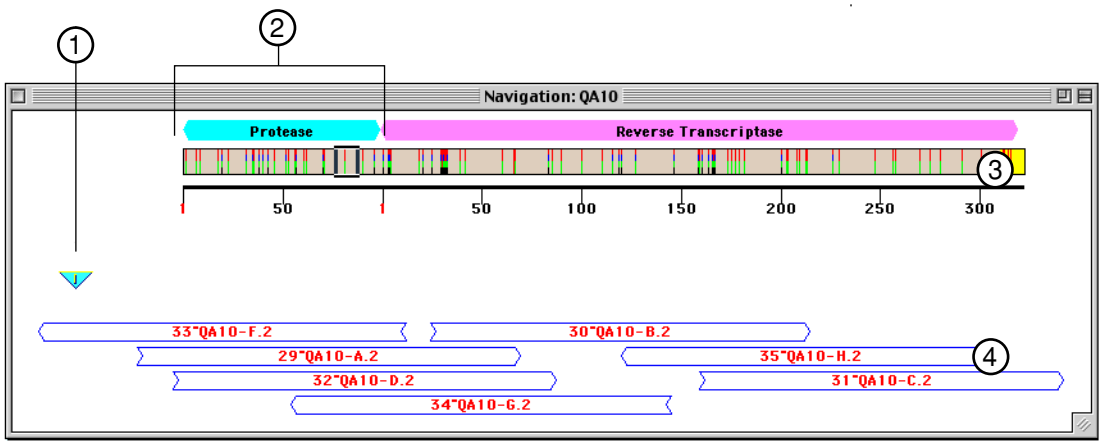
How You Can Use This Window

The following table describes actions you can perform from this window:

You can...	For more information, see...
add and remove segments from the project	♦ “Adding Segments to a Project” on page 7-24. ♦ “Removing Segments from a Project” on page 7-25.
Select an area of interest in the Navigation bar and jump to the area of interest in the **View Edit** window.	page 7-21.

Navigation Window Example

The following figure shows an entire assembled project displayed in the Navigation window.



Features of the Navigation Window

The following table describes the callouts in the “Navigation Window Example” above.

Navigation Window Features

Page 7-21 Callout	Feature	Description
1	Turn Triangle	Clicking the turn triangle toggles between the option of: ♦ Displaying a graphical representation of the sequence segments’ alignment. ♦ Not displaying the graphic.

Navigation Window Features *(continued)*

<table>
<tr><th>Page 7-21
Callout</th><th>Feature</th><th>Description</th></tr>
<tr><td>2</td><td>Vertical colored bars indicate positions of interest

They are represented by color-coded vertical lines on the Navigation bar.</td><td>A position of interest is any base in the consensus that is:

♦ Different from the HIV-1 pNL4-3 reference strain

♦ Found in the lookup table of known resistance positions.

Resistance mutations are those mutations reported to confer resistance to a specific anti-viral drug. The HIV-1 Genotyping System Software contains an internal table that lists all of the resistance mutations known at the time of release. This table is derived from the Los Alamos HIV database and is updated annually.

♦ Designated as mixed base in at least one segment at that position.

♦ A deletion relative to the reference sequence

♦ An insertion at that position.

♦ Identified to have mismatches between the segments.

The default is to have all of the feature selections chosen.

<table><tr><th>To...</th><th>See...</th></tr><tr><td>change the position of interest criteria use the Feature Selection window</td><td>“Setting Active Positions of Interest” on page 7-34.</td></tr></table></td></tr>
</table>

Navigation Window Features *(continued)*

<table>
<tr><th>Page 7-21
Callout</th><th>Feature</th><th>Description</th></tr>
<tr><td>3</td><td>Navigation Bar

Note A consensus position can be a position of interest based on more than one criterion.</td><td>A graphical representation of the assembled sequence with an amino acid numbering scale.

The positions of interest are displayed within the graphic.

The following table defines the colors within the assembled/consensus sequence graphic:
<table><tr><th>Color</th><th>Meaning</th></tr><tr><td>gray</td><td>Background (no differences relative to the reference)</td></tr><tr><td>red</td><td>Active position of interest

Note A red line from top to bottom in the navigation bar denotes an insertion.</td></tr><tr><td>black</td><td>Mismatches between segments</td></tr><tr><td>green</td><td>Known or unknown variant between reference and consensus</td></tr><tr><td>blue</td><td>Multibase position</td></tr><tr><td>yellow</td><td>Shows where the assembled consensus uses data from only one sequence segment or no sequence segments.</td></tr></table></td></tr>
<tr><td>4</td><td>Alignment View</td><td>The sequence segments graphic displays the sequence segments in a project labeled with the name and showing overlap.

Verify that the location and orientation of the segments are similar to the figure in the “Navigation Window Example” on page 7-21.

The segments are depicted as follows:
<table><tr><th>Feature</th><th>Meaning</th></tr><tr><td>Right arrow</td><td>Forward sequences (generated with primers A–D)</td></tr><tr><td>Left arrow</td><td>Reverse sequences (generated with primers F–H)</td></tr></table></td></tr>
</table>

Adding Segments to a Project

About Adding Segments to a Project

In certain situations, you may find it necessary to add or remove individual segments to a project. Adding segments in this way allows the user to specify which files are to be used in generating the project.

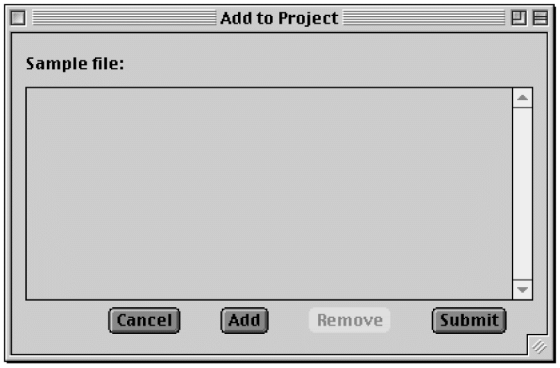
Typically you will add or remove segments manually if:

- The original sample name was incorrect.
- A segment sequence was repeated to improve data quality.
- The location of the segment was incorrect (*e.g.*, the start and stop points were incorrectly set so that the read length was inappropriate).

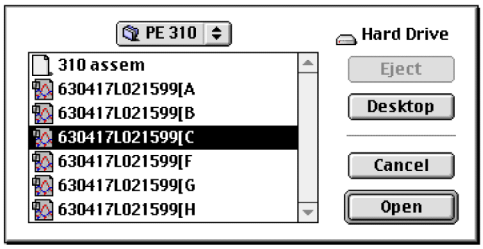
Procedure

Note When adding segments to a project, be careful not to add a sequence data file that is already part of the project.

To add segments to a current project:

Step	Action
1	Open the project to which you will add the segment.
2	Select **Add Segment** (⌘-A) from the **Edit** menu. The following window appears (see below). 

To add segments to a current project: *(continued)*

Step	Action
3	Click the **Add** button. The standard Macintosh computer navigation box appears. 
4	Navigate to the folder that contains the sample files you want to add.
5	You can either: ♦ Select the file and click **Open**, or ♦ Double-click the selected file The sample file is listed in the text area of the **Add to Project** window.
6	Repeat step 5 as needed.
7	Click **Submit**. The progress window appears, describing the process of adding the segment to the project.

Removing Segments from a Project

About Removing Segments from a Project

It may be necessary to remove a segment from a project if:

- Excessive mismatches cannot be handled by trimming. In this situation, you may want to repeat the sequencing for that segment and then manually add it to the project as described above (see “Adding Segments to a Project” on page 7-24).
- The original sample name was incorrect.

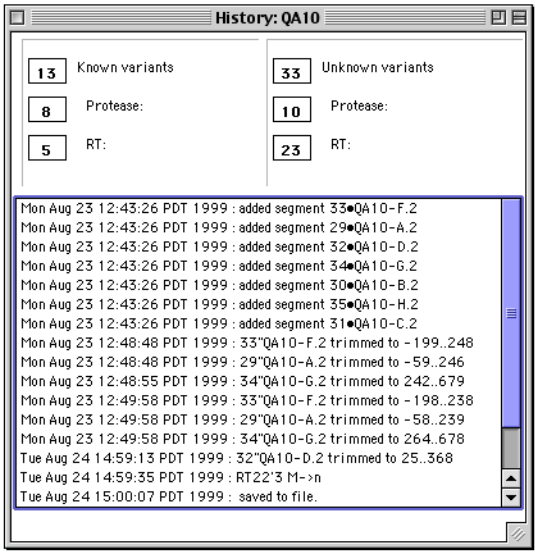
Procedure

To remove segments from a project:

Step	Action
1	In the **Alignment** View, click the segment that you want to remove. The segment becomes highlighted with a black bold line.
2	Select **Remove Segment** (⌘-R) from the **Edit** menu. The progress window appears, describing the process of removing the segment from the project. The segment is removed from the graphic and the project.

The History Window

The History window displays a current summary of the number of known and unknown nucleotide variants relative to the reference sequence. Subtotals of the number of known and unknown variants in the protease and RT genes are also shown. These numbers are updated only when changes are saved to the Project file.



Showing a Log of All Edits

When you have edited the consensus sequence, select Save from the Edit menu.

The History window shows a permanent record of segments that were added, removed, rejected, trimmed, or edited.

Note The large dot seen after lane number in the Data Collection software and the DNA Sequencing Analysis software is converted by the HIV-1 Genotyping System Software to straight quotes (").

Editing the Sequence

About This Step

Once the project has been assembled and you have reviewed it in the Navigation window, the next step is to review and edit the data.

Consensus Sequence Defined

A consensus sequence is calculated from the base assignments in each of the sequence segments. It may include mixed base positions and deletions, and reflect differences among segments.

Cursor

The following table describes how to show the cursor line:

To...	Then...
show the cursor line in the **View Edit** window	select **Toggle Position Cursor** (⌘-T) from the **Edit** menu.
turn the cursor line off	select **Toggle Position Cursor** again.

Overview of the Editing Procedure

To edit the sequence:

Procedure	See Page
Trim the ends of each segment, if needed, to remove poor quality base calls.	“Trimming Sequence Segments” on page 7-33.
Verify multibase positions.	“Setting Active Positions of Interest” on page 7-34.
Reconcile mismatched bases (those bases in overlapping segments that do not agree with each other).	“Editing Bases” on page 7-35.

Editing the Consensus Sequence Using the View Edit Window

The Editing Process

Trimming and base editing are carried out using the features of the View Edit window as follows:

Step	Action	See Page
1	Trimming Sequence Segments.	7-33
2	Edit positions of interest to generate an edited consensus sequence.	7-35

Before Manually Editing the Project

Before manually editing the HIV-1 Genotyping System Software project, make sure that in the DNA Sequencing Analysis Software the:

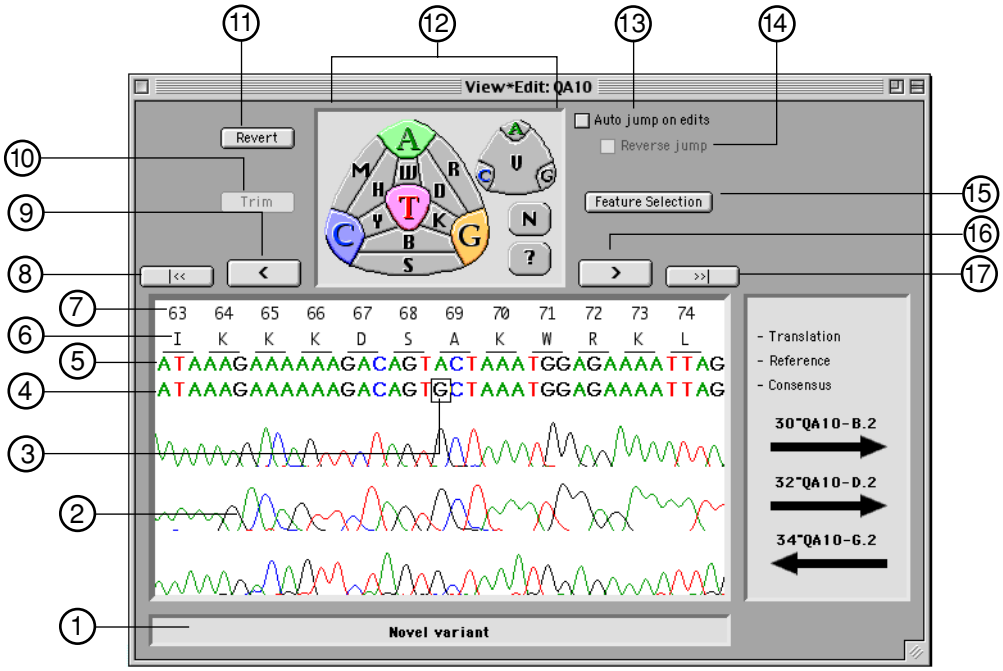
- Tracking is correct.
- Start and stop points are correct.
- Sample naming is correct.
- Matrix is appropriate for the analysis.

Displaying the Window

To display the View Edit window, double-click anywhere in the navigation bar in the Navigation window.

For an example of the Navigation window, see page 7-21, and for a description of the bar, see callout 3 on page 7-23.

Window Example The following is an example of the View Edit window:



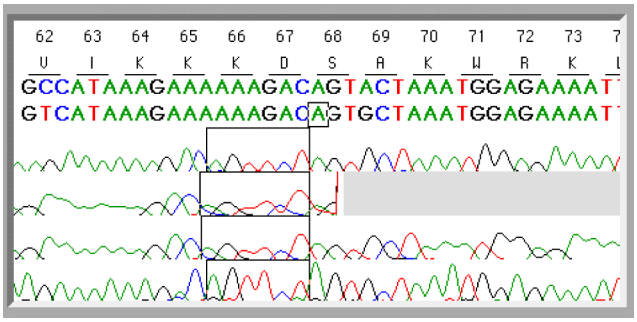
Features of the View Edit Window

The following table describes the callout numbers in the previous figure:

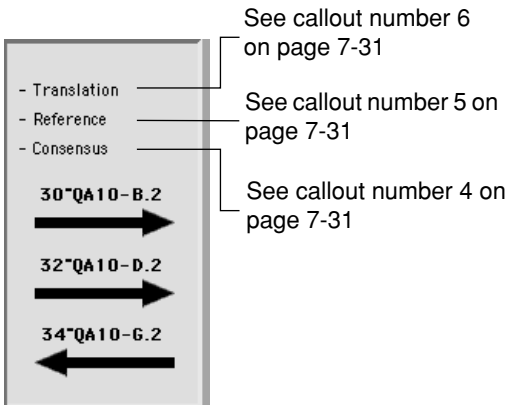
Callout number	Feature	Description
1	Position Information box	Explains why a position in the consensus has been identified as a position of interest.

The following table describes positions of interest and their definitions:

Item	Definition
Novel Variant	Change in the sequence that has not been reported as a resistance mutation in the Los Alamos HIV database
Reported Variant	A variant that is reported in the Los Alamos HIV database
Mismatch	When two or more segment sequences do not agree at a given position
Mixture	More than one nucleotide present at a single position
Indel	A base insertion or deletion in a sequence that cannot be aligned with other segments or with the HIV-1 pNL4-3 reference strain
Insertion	A set of three or multiple of three bases that cannot be aligned with the HIV-1 pNL4-3 reference strain, but that are present in more than one segment and are believed to have biological significance. While the consensus/reference sequence will be aligned and not showing the insertion, the segments will show the insertion and they will not be in alignment with the consensus/reference sequence. The box, shown in electropherograms in the **View Edit** window (see below), represents the sequence that is not shown in the consensus/reference sequence.



Note The insertion box is controlled by the **Toggle Position Cursor** (⌘-T) from the **Edit** menu. If the **Toggle Position Cursor** command is off, then the insertion box is not visible.

Callout number	Feature	Description
2	Segment electropherogram	Electropherogram of a sequence segment. The name and orientation of the segment is shown in the box to the right of the segment electropherogram. An example is shown below.  See callout number 6 on page 7-31 See callout number 5 on page 7-31 See callout number 4 on page 7-31
3	Position of interest (currently active)	Designates the currently active position of interest according to the current feature selections.
4	Consensus sequence	Sequence calculated from the base assignments in each of the sequence segments. It may include mixed base positions and deletions, and reflect differences among segments.
5	Reference sequence	The sequence of the HIV-1 pNL4-3 reference strain.
6	One-letter amino acid code **Note** When two (or more) amino acids are displayed in the translation information for one codon, this is displayed in the printed report as: A1 PositionN A2, A1 PositionN A3; that is, L33I, L33V. A = Amino Acid	Identifies all possible amino acid translations of each codon in the consensus sequence.
7	Codon number	Identifies the codon of the gene you are editing.
8	Jump to leftmost position of interest ()	Click this button to move the cursor to the leftmost position of interest.

Callout number	Feature	Description
9	**Reverse Jump** button (<)	Click this button to move the cursor to the previous position of interest. You may also use the Reverse Arrow (←) key on the Macintosh computer expanded keyboard.
10	**Trim** button (Trim)	See “Trimming Sequence Segments” on page 7-33.
11	**Revert** button (Revert)	Reverts the active position of interest in the consensus sequence to the original base assignments, regardless of any manual edits performed.
12	IUB code buttons/Editing Palette window	Use to edit the consensus sequence. For a list of the International Union of Biochemists (IUB) codes, see Appendix E, “IUB Codes.”
13	**Auto jump on edits** checkbox (☐ Auto jump on edits)	See “Moving Among Positions of Interest” on page 7-36.
14	**Reverse jump** checkbox (☐ Reverse jump) **Note** This is only active when the checkbox labeled “Auto jump on edits” is selected.	
15	**Feature Selection** button (Feature Selection)	Click this button to display the **feature selection** window. **For more information on...** — **See Page** using the **Feature Selection** window to set active positions of interest — 7-34
16	**Forward Jump** button (>)	Click this button to move the cursor to the next position of interest. **Note** You may also use the Forward Arrow (→) key on the Macintosh computer expanded keyboard.
17	Jump to the rightmost position of interest (>>|)	Click this button to move the cursor to the rightmost position of interest.

Trimming Sequence Segments

About Trimming Sequences

The HIV-1 Genotyping System Software automatically trims data at the ends of the segments based on recognition of the sequence segments being analyzed. Regions of an electropherogram that have been trimmed are indicated by a gray background. Trimmed regions remain visible but are not used to calculate the consensus sequence.

About Manual Trimming

There are certain situations in which you may wish to manually modify the trimmed regions. Manual trimming is normally performed when the data quality of one segment is causing excessive mismatches. By trimming that region of the segment, the number of mismatches can be minimized.

To Manually Trim Regions

Step	Action
1	Click the position in the electropherogram where you want to start the trimming procedure. The inverted selection extends either to the right or to the left, depending on which end of the segment is closest, and trims the sequence to the nearest end. **Note** To undo the trim selection, drag it off the window using the mouse.
2	Click the **Trim** button. A progress dialog box appears.

About Manually Untrimming

Untrimming is the process of changing the region of sequence that the HIV-1 Genotyping System Software has automatically trimmed.

If...	Then...
you feel that too much sequence has been trimmed automatically, and if the data quality for that region is acceptable	you can manually change the trimmed region.

To Manually Untrim Regions

Step	Action
1	Click on the portion in the electropherogram where you want the trimmed region to end. The portion of the trimmed electropherogram that is not selected will remain trimmed. The deselected portion that is gray will be white and untrimmed.
2	Click the **Trim** button.

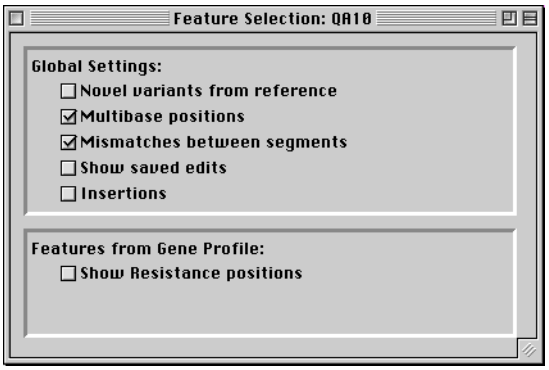
Setting Active Positions of Interest

What Are Active Positions of Interest?

Active positions of interest are the only positions in the project that can be edited. These positions are defined by the user in the Feature Selection option of the View Edit window.

Procedure

To set active positions of interest:

Step	Action
1	<table><tr><th>You can either...</th><th>Result</th></tr><tr><td>click the **feature selection** button in the **View Edit** window.</td><td rowspan="2">The **feature selection** window appears.</td></tr><tr><td>select **Analysis** from the **Window** menu.</td></tr></table> 
2	Make the check boxes of your choice.
3	Deselect all the other checkboxes.

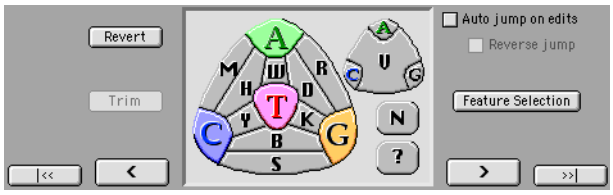
To set active positions of interest: *(continued)*

Step	Action
4	Click the Close box to close the window. In the assembled sequence graphic of the **Navigation** window, only those positions of interest corresponding to the above selections are identified. Active positions of interest are indicated by red vertical lines.

Editing Bases

If mismatches occur between overlapping segments, you will need to perform manual editing to reach agreement.

To edit the consensus base at a position of interest:

Step	Action
1	Click the position of interest.
2	In the **Editing Palette** window (see figure below), click the button that corresponds to the IUB one-letter code for the replacement base. **Note** You may also use the letters on the Macintosh computer keyboard to edit the sequence.  **Note** The IUB one-letter codes are listed in Appendix E, "IUB Codes."
3	Click the right arrow. This moves the current position of interest to the next active position of interest. **If...** / **Then...** you first select the **Auto jump on edits** checkbox / you do not need to click the right arrow. The cursor automatically jumps to the next position of interest once the edit has been made.

Moving Among Positions of Interest

The following are the cursor controls for moving among the positions of interest:

- Cursor Control Using the View Edit Menu
- Cursor Control Using the Navigation Window

Cursor Control Using the View Edit Menu

For more information, see “Editing the Consensus Sequence Using the View Edit Window” on page 7-28.

View Edit menu Cursor Controls

Control Feature on the View Edit menu	Take this action
Forward jump button (right arrow) 	Click to move to the next active position of interest to the right. **Note** You may also use the Forward Arrow (→) on the Macintosh computer expanded keyboard.
Reverse jump button (left arrow) 	Click to move to the next active position of interest to the left. **Note** You may also use the Reverse Arrow (←) on the Macintosh computer expanded keyboard.
Home arrow 	Click to move to the first (5´ most) active position of interest.
End arrow 	Click to move to the last (3´ most) active position of interest.
Auto jump on edits checkbox	**If...** you select this checkbox in the **View Edit** window **Then...** the cursor moves to the next position of interest to the right after you click the IUB code button for the replacement base.

View Edit menu Cursor Controls *(continued)*

<table>
<tr><th>Control Feature on the View Edit menu</th><th>Take this action</th></tr>
<tr><td>Reverse jump checkbox</td><td>
<table>
<tr><th>If...</th><th>Then...</th></tr>
<tr><td>you select this checkbox in the View Edit window</td><td>the current position of interest automatically jumps to the next position of interest to the left.</td></tr>
</table>
Note This works only when the checkboxes labeled Reverse jump and Auto jump on edits are selected.

This occurs after you click the IUB code button for the replacement base.
</td></tr>
</table>

Cursor Control Using the Navigation Window

For more information, see “Features of the Navigation Window” on page 7-21.

Control Feature	To...	Then...
Navigation Bar	move the current position of interest to that location	click on a position of interest in the assembled sequence graphic.

Reconciling Segment Mismatches

About Reconciling Segment Mismatches

The following table describes how to reconcile segment mismatches:

To...	Then...
reconcile segment mismatches after editing the consensus sequence	a. Examine the consensus sequence in more detail. b. Adjust the active positions of interest. The active positions of interest are set using the **feature selection** window. Active positions of interest are those that are available for editing.

Analysis Options

The following table describes the analysis options:

Description of Analysis Options

Option	Description
Novel variants from reference	Locates unreported differences relative to the reference.
Multibase positions	Allows you to go to the consensus sequence positions that are derived from segments that have more than one nucleotide at a position.
Mismatches between segments	Locates differences in a consensus sequence position that is derived from segments that do not agree at that position.
Show saved positions	Locates bases that were manually edited and saved in the project.
Insertions	Locates extra bases that code for amino acid(s) that cannot be aligned with the HIV-1 pNL4-3 reference strain.
Show Resistance positions	Locates positions in the consensus sequence that are found in the resistance mutations table (see “Resistance Mutations” below).

Resistance Mutations

Definition of Resistance Mutations

The mutations that are listed in the Los Alamos database (Korber *et al.*, 1977) have been reported to confer resistance to a specific antiviral drug. The HIV-1 Genotyping System Software contains an internal table that lists all of the resistance mutations known at the time of the software release. This table will be updated annually.

Changing Positions of Interest to Review in the Report

To...	Then...
review only those positions that will change the genotyping results of the analysis on the report	in the **Feature Selection** window (see "Setting Active Positions of Interest" on page 7-34): ♦ Uncheck all selections in the **Global Settings** section. ♦ Check the **Show Resistance positions** checkbox in the Features from **Gene Profile** section. For information about printing the report, see page 7-41.

Saving Projects

Formats in Which to Save Projects

From the File menu you can save projects in the following formats:

Choose...	To...
Save (⌘-S)	save the manual edits made in the project.
Save FASTA (⌘-F)	save the assembled sequence in the standard FASTA format. Many bioinformatics programs can read this format. The FASTA files are saved to the **Report** folder located in **Projects:Completed** folder.
Save Genotype (⌘-G)	save the assembled sequence in a tab-delimited format that can be imported into spreadsheets or database programs. The genotype files are saved to the **Report** folder located in **Projects:Completed** folder.

Printing a Report

About the Report

Generate a project report after you have edited and reviewed the consensus sequence. The report summarizes the genotype and quality control information for the project.

IMPORTANT Before printing a final report, examine the quality standards in Appendix A.

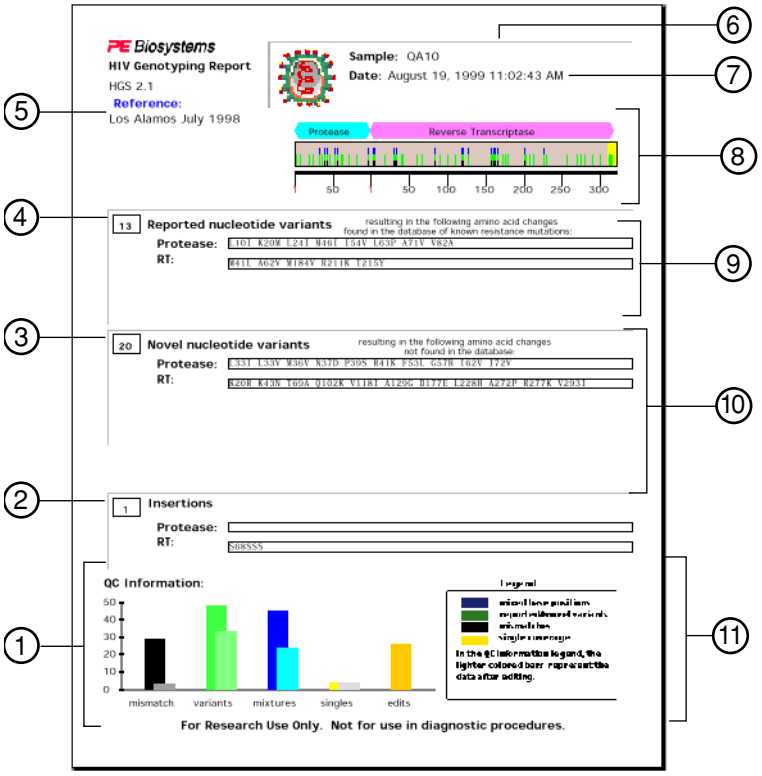
Printing the Report

Note Before printing, you must select a printer from the Chooser.

From the File menu, select Print Report (⌘-P), and click Print when the Print dialog box appears. The report is not displayed on the computer screen.

Report Example

An example of a report is shown next.



Report Description

The following table describes the contents of each area in “Report Example”:

Callout number	Heading
1	Quality Control Information. In each pair of histogram bars, the left bar represents the number before editing and the right bar is the total number after editing.
2	Insertions Total number of nucleotide insertions.
3	Novel nucleotide variants. Total number of nucleotide changes that result in novel amino acid variants that are not reported in the Los Alamos HIV database.
4	Reported nucleotide variants. Total number of nucleotide changes that result in amino acid variants that are reported in the Los Alamos HIV database.
5	HIV-1 Genotyping System Software version number and the version of database.
6	Sample Name.
7	Date the report was printed.
8	Summary of project consensus sequence.
9	Reported variants at the amino acid level.
10	Novel variants at the amino acid level.
11	Quality Control Information legend.

Heading	Description
Mismatches	Nucleotide bases in a segment that do not agree with the bases in the overlapping segment(s).
Variants	Amino acid changes resulting from nucleotide changes.
Mixtures	More than one nucleotide detected at a single position, due to a mixture of viral strains.
Singles	Positions of interest in single coverage area.
Edits	Total number of edits performed.

Setting AutoLaunch

About AutoLaunch

The AutoLaunch folder contains the AutoLaunch application. AutoLaunch is an independent program that allows the HGS software to create projects automatically from sample files in a run folder.

Note Using the AutoLaunch application is optional.

How to Use AutoLaunch

Start this application, and leave it running. The AutoLaunch program must be running to launch the analysis. You can specify a time each day for the AutoLaunch program to review the contents of a run folder to determine if new sample files have been generated from which projects can be created. The AutoLaunch program is particularly useful if you have performed an overnight sequencing run because it can create projects that will be ready for review.

Automatically Starting AutoLaunch

If you make an alias of the AutoLaunch application and put the alias in your startup folder, when you turn on your Macintosh computer the application will start.

To make an alias of the application, select the icon and click ⌘-M. Put the alias in your Startup Items folder, which is located in the System Folder.

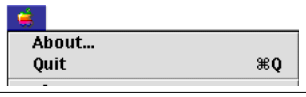
Setting Up the Application

To set AutoLaunch:

Step	Action
1	Open the **HIV Genotyping** folder, open the **AutoLaunch** folder, and take the following action: a. Place the folder titled **PutMeInYourRunFolder** in the **Run** folder. b. Double-click the **AutoLaunch** application, which has the following icon:  The following window opens. 
2	Navigate to the **Run** folder, and click **Open**. The **Run** folder is located in the **Collection Software** folder in the ABI PRISM® 377 and ABI PRISM® 310 Data Collection software.
3	Use the **Earlier** and **Later** buttons to adjust the hour.
4	Close the dialog box. The program is still running. AutoLaunch runs as follows: a. Polls the folder at the set time, waits 5 minutes before polling the folder again to ensure that the folder is stable, before to launching the HIV-1 Genotyping System Software. b. The application sets the polling time for one day later and runs in the background.

Reopening or Quitting AutoLaunch

The following table explains how to reopen and quit the AutoLaunch application:

To...	Go to...
reopen the AutoLaunch window	the **Apple** menu and select **About**... 

To...	Go to...
quit the AutoLaunch application	the **Apple** menu and select **Quit** (⌘-Q).
To...	**See...**
create projects manually in the HIV-1 Genotyping System Software	“Creating a New Project” on page 7-17.

Before Manual Editing of the Projects

Verify the following:

- The tracking is correct.
- The start and stop points are appropriate.
- The samples are named properly.
- The matrix from the correct instrument is used.

8 Troubleshooting

Troubleshooting Table

How to Use Use the following troubleshooting table to diagnose and solve problems.

The troubleshooting recommendations are based on the assumption that all kit reagents are stored according to their manufacturers' specifications and that the directions in this manual have been followed correctly.

Troubleshooting Table

<table>
<tr><th>Observation</th><th>Possible Causes</th><th>Recommended Action</th></tr>
<tr><td>Presence of precipitate in Viral Lysis Buffer.</td><td>Storage temperature is too low.

Performance is not affected once the precipitate is redissolved.</td><td>♦ Warm the buffer in a 37 ˚C water bath until the precipitate is dissolved.
♦ Store at 2–8 ˚C.</td></tr>
<tr><td>♦ PCR product for a sample is not visible on the agarose gel.

But

♦ Positive Control shows a clear band.

And

♦ Mass ladder is clear and well defined.</td><td>Not enough RNA in RT-PCR.</td><td><table><tr><th>If the sample was...</th><th>Then...</th></tr><tr><td>resuspended in 100 µL Sample Diluent</td><td>Repeat DNA purification step, but resuspend RNA in 50 µL Sample Diluent.</td></tr><tr><td>resuspended in 50 µL Sample Diluent</td><td>Repeat RNA purification step, but this time start with 1 mL plasma.</td></tr></table></td></tr>
</table>

Troubleshooting Table *(continued)*

Observation	Possible Causes	Recommended Action
♦ PCR product for a sample is not visible on the agarose gel. But ♦ Positive Control shows a clear band. And ♦ Mass ladder is clear and well defined.	Residual ethanol supernatant was left in the tubes during the RNA washing steps. Viral RNA pellet may have been lost after precipitation.	Carefully remove the ethanol supernatant, following the instructions at each step (see “Washing the RNA Pellet” on page 4-7). Allow ethanol to evaporate by leaving the tube cap open. The orientation mark on the tube should face the outside rim of the rotor during centrifugation, and the supernatant must be carefully removed from the side opposite the orientation mark (see “Precipitating Viral RNA” on page 4-6).
♦ PCR product for a sample is not visible on the agarose gel. And ♦ PCR product for the Positive Control is not visible. But ♦ Mass ladder is clear and well defined.	Thermal cycler used for RT or PCR is not calibrated correctly.	Check calibration of the thermal cycler.
	RNase contamination leading to degradation of RNA.	♦ Decontaminate your work space and tools. ♦ Use fresh reagents. ♦ Repeat RT-PCR with positive control.
	Poor yield of PCR product after Microcon® purification.	♦ Repeat RT-PCR. Load product onto an agarose gel before Microcon purification. If smear is seen at approximately 100 bp, amplicon contamination of PCR may be suspected. ♦ Make sure that the thermal cycler was preheated to 42 ˚C before inserting the reaction tubes for the RT reaction. ♦ Make sure that the RT reactions were set up on ice or on a cold block.

Troubleshooting Table *(continued)*

Observation	Possible Causes	Recommended Action
♦ A 1.8 kb PCR band with an intensity greater than the 2.0 kb mass ladder band. And ♦ Nonspecific bands on the agarose gel, which are >10% of the 1.8 kb PCR band. But ♦ RNA control has a clear and well-defined band. And ♦ Mass ladder is clear and well defined.	Too much RNA input into the RT-PCR.	♦ Dilute RNA 1:10 with RNA Diluent, then repeat RT-PCR.
All of the following: ♦ RT-PCR bands appear smeared on the agarose gel. ♦ RNA control bands appear smeared. ♦ Mass ladder is clear and well defined.	Reagents and/or samples were not kept at 2–6 ˚C until the start of the RT reaction. PCR product samples were heated at high temperature, then quickly cooled resulting in denatured PCR product.	♦ Keep all samples and reagents on ice. ♦ Move tubes quickly to the thermal cycler, which is prewarmed to 42 ˚C. ♦ Reheat samples, then slowly cool to allow reformation of double-stranded DNA.
Any of the above conditions, when the mass ladder is either absent or abnormal in any way.	Poor agarose gel conditions.	♦ Use new agarose, running buffer, loading buffer, and ethidium bromide. ♦ Rerun the agarose gel.

Troubleshooting Table *(continued)*

Observation	Possible Causes	Recommended Action
Sequencing electropherogram fluorescence intensity is too low (<100).	♦ The concentration of the sequencing template is below the range of the assay. ♦ The sample was lost during ethanol precipitation.	♦ If sufficient PCR product remains, repeat the sequencing reactions. ♦ If necessary, repeat the RT-PCR reaction and Microcon purification. ♦ Use a lower dilution of the PCR product and repeat the sequencing reactions.
		Make sure that the absolute ethanol you used is fresh. If in doubt, start again with a new bottle.
		Make sure that the proper volume of 3 M sodium acetate was added to the sodium acetate/EtOH mixture used for the precipitation steps. Thorough mixing at this step is very important.
		Make sure that you do not disturb the pellet when aspirating supernatant.
		After resequencing the sample, resuspend the dried pellet in 3 µL (rather than 5 µL) Formamide:Loading Buffer.
		Run a BigDye sequencing standard (P/N 4304154) to verify: ♦ Gel quality. ♦ That the instrument is operating within specifications.
	Centrifugation forces for the precipitation steps were incorrect.	Follow instructions for the precipitation steps exactly as written.
Sequencing electropherogram fluorescence intensity is too high (peak tops are cut off, peaks are too wide at the beginning of the run).	The concentration of the sequencing template is above the range of the assay.	♦ Make a larger dilution of the PCR product prior to sequencing. ♦ Repeat the sequencing reactions.
Dye blobs are present at the beginning of the sequence, obscuring the electropherogram peaks.	Incomplete removal of unincorporated BigDye™ Terminators.	Make sure that you adequately vortex samples during the washing steps.

Troubleshooting Table *(continued)*

Observation	Possible Causes	Recommended Action
Noise in the sequencing data.	Residual ethanol supernatant was left in the reaction during pelleting and washing steps.	Carefully remove the ethanol supernatant, following the instructions at each step.
	Poor matrix.	♦ Make another matrix each time your instrument has been serviced, especially if adjustments were made to the optical system. ♦ Run a BigDye™ sequencing standard (P/N 4304154) to verify gel quality.

Quality Standards A

Introduction

The quality of your genotyping data depends on many factors. Before producing a final report, examine the quality standards described below and evaluate whether your report will meet these standards.

Quality of the Primer Sequence

A sequence file from an individual primer should pass these quality standards:

- Data was analyzed using the ABI100 basecaller (ABI PRISM® 377 DNA Sequencer) or the ABI CE-1 (ABI PRISM® 310 Genetic Analyzer).
- Data was analyzed with DNA Sequencing Analysis software version 3.1 to 3.3.
- The peak spacing is between 9.1 and 14 (inclusive).

Reasons for Repeating the Sequencing

Repeat the sequencing from the appropriate primer if there is:

- Disagreement in any base call between forward and reverse primers that cannot be reconciled. If there is any disagreement, repeat the forward and reverse primer reactions.
- The presence of one or more insertion or deletion in the sequence of one primer that have not been verified by the opposite sense primer.

Quality of the Consensus Sequence

The characteristics of a high quality consensus sequence are that:

- There are no unresolved ambiguities (Ns).
- There are data from sequences in both orientations for each base reported in the consensus, except at the 3´ -most end and the 5´ -most end, if only the D primer sequence is used in the consensus.
- Base mixtures are verified by sequencing in both orientations. In at least one orientation:
  - The smaller peak should be at least 30% of the maximum peak height.
  - Both peaks should be clearly visible in the second orientation but do not have to meet the 30% criteria.
- All the individual sample files used to generate the consensus meet the individual quality criteria.

Preventing RNA Degradation

B

Introduction

To be successful with this genotyping procedure, it is essential to prevent degradation of your RNA samples.

How RNA Is Degraded

RNA is degraded by RNases. These enzymes occur naturally in cells and are liberated during cell lysis. RNases can remain in samples derived from cells if purification is not complete. In addition, RNases can be introduced into samples by contact with surfaces that we have touched. This is because our skin secretes RNases.

RNases are very stable and do not need any cofactors, so they linger on surfaces and remain functional under a wide range of environmental conditions. They have a high activity, so only a small amount of contamination can cause significant loss of a sample of RNA.

Sources of Contamination

Sources of RNase contamination include:

- General laboratory glassware and plasticware
- Hands
- Contaminated solutions

Precautions

Follow these precautions during the genotyping procedure to help prevent RNA degradation (Sambrook *et al.*, 1989).

- Purchase gel solutions from a MLS which has analyzed solutions for the absence of RNases and DNases.
- Use only new sterile plasticware.
- Keep separate stocks of chemicals for use with RNA only.
- Keep a selection of glassware, plasticware, and chemicals for use with RNA sample preparation only.
- Bake all glassware and metal spatulas in an oven at 300 °C for at least 4 hours.
- Wear gloves at all times and change them frequently.
- Select plasticware that is resistent to chloroform and either:
  - Rinse with chloroform.

! WARNING ! CHEMICAL HAZARD. Chloroform is extremely toxic and a potential human carcinogen. This chemical is highly corrosive to skin and eyes. Please read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

  - Treat all water and salt solutions with diethylpyrocarbonate (DEPC), which inactivates RNases. (Solutions containing EDTA cannot be treated with DEPC).

To treat solutions:

Step	Action
1	Add DEPC to a concentration of 0.2% (v/v). **CAUTION CHEMICAL HAZARD. Diethylpyrocarbonate (DEPC) is a combustible liquid and vapor. It may be harmful if swallowed or inhaled. Exposure may cause irritation to the eyes, skin and respiratory tract. Please read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.**
2	Store for at least 12 hours at 37 °C.
3	Heat to 100 °C for 15 minutes or autoclave for 15 minutes at 15 lb/sq. in. on the liquid cycle.

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Amino Acid Codes D

The following table lists the amino acids, and the corresponding three-letter and one-letter codes.

Amino Acid	Three-Letter Code	One-Letter Code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartate or Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamate or Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

IUB Codes

E

The following table lists the International Union of Biochemists (IUB) codes and complements:

IUB Codes		Complements			
A = adenosine	S = G or C (Strong—3 H bonds)	A	T, U	R	Y
C = cytidine	W = A or T (Weak—2 H bonds)	C	G	Y	R
G = guanosine	Y = C or T (pYrimidine)	G	C	K	M
T = thymidine	B = C, G, or T	T	A	M	K
U = uracil	D = A, G, or T	U	A	S	W
K = G or T (Keto)	H = A, C, or T	D	H	W	S
M = A or C (aMino)	V = A, C, or G	H	D	B	V
R = A or G (puRine)	N = aNy base	N	N	V	B

Glossary

This glossary includes some of the special terms used in this manual. If a special term is not defined here, check the index to see if it is explained elsewhere in the manual.

Assembled Sequence	A sequence that is assembled from individual sequence segments but which has not been edited to make a consensus sequence.
Consensus Sequence	A sequence that results after the assembled sequence is edited.
Crucial Position	A base that if changed will change the genotypic assignment.
Discrepancy	The state that exists when the consensus sequence does not agree with the reference sequence.
Edited Position	A base that has been edited to produce the consensus sequence.
Insertion	Additional groups of 3 bases that cannot be aligned with the HIV-1 pNL4-3 reference strain, but that are found in all segments and are believed to have biological significance.
Mismatch	The instance when two or more segment sequences do not agree.
Mixture	The state of more than one nucleotide showing at a single position in a segment.
Multibase Position	A base position that the DNA Sequencing Analysis software basecaller has been unable to call as a single, specified base.
Novel Variant	A change in the sequence that produces an amino acid change that is not associated with a resistance mutation reported in the Los Alamos HIV database.
Project	The sequence segments, assembled sequence, and consensus sequence that are all part of a single genotyping analysis.

Resistance Position (Known Variant)	A base position that is known to produce an amino acid change that confers resistance to one or more particular anti-HIV drugs.
Reported Variant	An amino acid variant that is reported in the Los Alamos HIV database.
ACTG	Abbreviation for Aids Clinical Trials Group.
Segment	A single sequence derived from one primer and stored as one sample file.
Unknown Variant	A base position that is identified as an amino acid mutation that has not previously been documented.

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